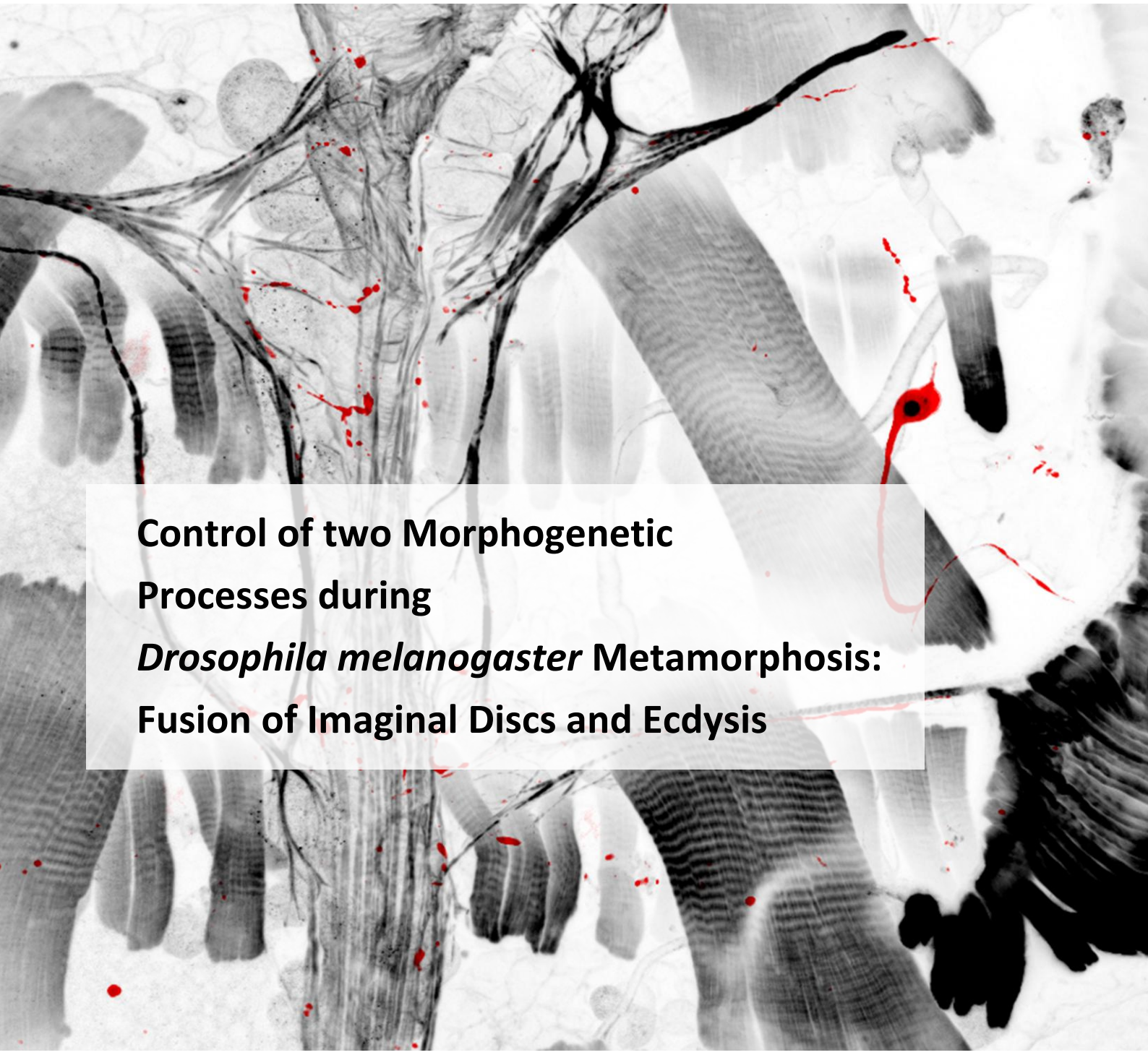
Universidad Autónoma de Madrid
Facultad de Ciencia
Departamiento de Biología Molecular



Control of two Morphogenetic Processes during *Drosophila melanogaster* Metamorphosis: Fusion of Imaginal Discs and Ecdysis

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Arthropods cuticle constitutes an external skeleton or exoskeleton. It has a protective function, such as support or defense against predator, but also has a physiologic role by preventing from desiccation or by sensing its environment among others. Arthropods suffer various molting throughout their lifetime. Their cuticle is highly rigid and has to be periodically shed to allow the animal to grow or to change its form. This process highly stereotyped, referred as ecdysis, is followed by a postecdysis period which permits the cuticle to become mature.

***Drosophila melanogaster* is a holometabolous insect. Its life cycle starts with the fecundated egg. At the end of the embryogenesis, the embryo hatches to give rise to a small size larva. Two molts are necessary to obtain the grown larva, which then stop moving. The cuticle of the larvae is then shed and used as a cocoon, called pupal case, where a complete metamorphosis (from the Greek *meta* "change" and *morphe* "form") occurs. At the final ecdysis (eclosion), the adult fly emerges. During the post-eclosion behavioral sequence the newly formed and soft cuticle get tanned, that is to say get hardened and darken, and the wings extend.**

Adult thorax derives from a couple of larval structures, which are of ectodermal origin, called wing imaginal discs. During the metamorphosis, they migrate dorsally and fuse at the midline in a two slide fasteners manner similar to a wound healing. During this Thesis, we studied the implication of the Hox gene *Antennapedia* (*Antp*) during thorax closure. We found that *Antp* expression is restricted to the cells that initiate this morphogenetic event and alteration of its expression leads to a split thorax. Our results indicate that this phenotype is associated to a missregulation of JNK signaling which in turn affects actin cytoskeleton organization and filopodia formation needed in the leading edge cells for tissue migration.

We also focus our interest on a later *Drosophila* developmental stage, the post-eclosion. The mechanism underlying the post-eclosion sequence is known to be controlled by the neuropeptide Bursicon, which is synthesized by a subset of neurons called CCAP. We found that the transcription factor Opa is expressed in the CCAP cells and that downregulation of *opa* expression mimics the phenotype of alteration in *Bursicon* expression. Our data point to a requirement of Opa expression in the CCAP cell to prevent them from apoptosis. Besides, we identified Opa as the first uncovered positive regulator of *Bursicon* expression.

Presentación

Introducción

Drosophila es un insecto holometábolo, es decir, efectúa una metamorfosis completa. Su desarrollo se define en cuatro estadios: una fase embrionaria seguida de una fase larvaria, una pupal y una adulta. La transición entre cada fase se conoce como ecdisis y permite al animal tanto crecer de tamaño, dado que tiene un exoesqueleto, como cambiar de forma. En la mosca, la hormona ecdisona es responsable de estos cambios fisiológicos.

La cutícula adulta deriva de unos grupos de células llamado discos imaginales que se especifican durante la embriogénesis y se desarrollan durante los estadios larvarios. En la metamorfosis, la parte central de los discos se desinvaginan dando lugar a un apéndice mientras que la región periférica da lugar a la pared corporal. De hecho, la parte más proximal de cada disco tiene que fusionarse con la parte proximal de los discos vecinos para formar un cuerpo continuo. Los discos se llaman en función del órgano adulto principal al que dan lugar. Así, el disco imaginal de ala da lugar al ala propia, pero también a la axila y a la mitad del notum adulto. Durante la metamorfosis, el par de discos imaginales de ala se evierten bajo control de la hormona ecdisona, y las partes próximo-dorsales de los mismos (el tallo) se dirigen hacia la línea media para fusionarse. Este proceso morfogenético se llama cierre del tórax y ocurre entre las 5 y 7 horas tras la entrada en el estadio pupal, implicando una pérdida de la polaridad apico-basal de las células del tallo, además de una

reorganización del citoesqueleto de actina y de la emisión de filopodios. Estos cambios de estructura de la célula facilitan la migración de los discos imaginales hacia la línea media, utilizando la epidermis de la larva como sustrato para ese movimiento, y el reconocimiento de las células de ambos discos imaginales para la fusión correcta de los mismos. Se han descrito dos vías de señalización implicadas en este proceso; la vía de Decapentaplegic (Dpp) y la vía de c-Jun N-terminal kinase (JNK). Los mutantes para los componentes de estas vías dan lugar a moscas con el tórax hendido.

Tras 12 horas de haber entrado en pupación, el individuo presenta una morfología similar al adulto. Los distintos discos imaginales se han fusionado totalmente y la cabeza, que originalmente se encontraba dentro del cuerpo, se ha sacado del tórax. Mientras, las contracciones musculares dan lugar a un pulso de la hemolinfa (líquido circulatorio) permitiendo la externalización de las diferentes partes del cuerpo. Un mecanismo similar ocurre tras la eclosión adulta, durante un periodo de 30 minutos, permitiendo la extensión de la nueva cutícula formada y la expansión de las alas. Además, durante las primeras 3 horas, la cutícula se endurece y adquiere su color final mas pigmentado gracias a la incorporación de compuestos fenólicos. Las neuronas “crustacean cardioactive peptide” (CCAP), localizadas en el sistema nervioso central, han sido identificadas como reguladores de la ecdisis debido a la síntesis del neuropéptido Bursicon (Burs). Este neuropéptido corresponde a una proteína heterodimérica con nodos de cistina. Cuando la larva entra en metamorfosis, Burs se libera en la hemolinfa y actúa a través de su unión a un receptor acoplado a proteína G, Rickets (Rk), dando lugar a un incremento del mensajero secundario Adenosín monofosfato cíclico (AMPc).

Objetivos

El primer objetivo de esta Tesis consiste en entender el papel del gen Hox *Antennapedia* (*Antp*) en el disco imaginal de ala. Esto implica describir su patrón de expresión en este disco en el individuo silvestre y analizar su función. Nuestros resultados muestran un requerimiento de Antp durante el cierre del tórax de la mosca adulta. Por último, hemos identificado la vía de señalización controladas por *Antp* en esta función del cierre del tórax.

El segundo objetivo de esta Tesis radica en exponer el papel del factor de transcripción Odd paired (Opa) durante la ecdisis adulta. Para ello, se requiere establecer una correlación entre

el patrón de expresión silvestre de Opa y las células CCAP, y entender la función de Opa en la regulación del neuropéptido Bursicon (Burs) que se expresa en las neuronas CCAP.

Resultados Capítulo I: El gen Hox *Antennapedia* (*Antp*) regula el citoesqueleto de actina durante el cierre del tórax adulto de *Drosophila.*

En el disco imaginal de ala, *Antp* se expresa principalmente en la parte proximal, siendo más fuerte su expresión en la parte anterior del notum presuntivo, denominado prescutum. Esta expresión de *Antp* depende del promotor *P1*.

Mediante el uso del ARN interferente (ARNi) contra el ARN mensajero (ARNm) de *Antp* hemos alterado la expresión de *Antp* específicamente en el dominio presuntivo del notum. Este experimento da lugar a un tórax hendido en la mosca adulta. Se ha verificado que este fenotipo no se debe a una posible muerte celular asociada a la falta de expresión de *Antp*.

Además, hemos identificado al gen Hox *Ultrabithorax* (*Ubx*) como un represor de la expresión *Antp* en el disco de ala, dado que la expresión ectópica de *Ubx* reprime la expresión de *Antp.* Del mismo modo, cuando se inhibe *Ubx* tiene lugar una des-represión de *Antp* en el epitelio peripodial. De acuerdo con estos datos, la expresión ectópica de *Ubx* en la células del tallo fenocopia la falta de *Antp*, dando lugar a la escisión del tórax adulto tras la metamorfosis.

La falta de cierre del tórax adulto se atribuye a una alteración en el reclutamiento de actina en las células del tallo y también a la falta de emisión de filopodios, los cuales son requeridos para el acercamiento de ambos discos de ala. Encontramos que estos defectos son en parte debidos a una alteración de la vía JNK pero no de la vía de Dpp.

Resultados Capítulo II: La proteína con dedos de zinc Odd paired (Opa) está implicada en la post-ecdisis adulta de *Drosophila* mediante la regulación del neuropéptido Bursicon.

Hemos observado que el factor de transcripción *opa* se expresa en las neuronas CCAP presentes en el sistema nervioso central al largo del desarrollo, desde la generación de dichas neuronas hasta el estadio adulto, de manera independiente del estadio hormonal de estas neuronas. También, se han identificado unas nuevas poblaciones de neuronas CCAP pertenecientes al sistema nervioso periférico que expresan *opa*.

La inhibición de la expresión de *opa* específicamente en las neuronas CCAP mediante la expresión de un ARNi específico da lugar a un fallo en el desarrollo durante la ecdisis adulta; en concreto a un fallo en la extensión de las alas y del tórax, y a la ausencia de esclerotización y pigmentación de la cutícula. Opa es el responsable de estos defectos al actuar a dos niveles: uno, en la viabilidad de estas neuronas, y dos, como regulador positivo de la expresión de *Burs*.

Tanto la sobreexpresión de *opa* como la expresión ectópica de *Zic2*, un homólogo de *opa* en vertebrados, son capaces de activar la expresión de *Burs* en las neuronas CCAP. Otro dato interesante es que Opa también pueda activar la expresión de *Burs* en neuronas diferentes a las CCAP. Además, la alteración de la expresión de *Opa* o de *Zic2* afecta tanto a la ecdisis pupal como a la adulta.

Previamente se había identificado que los músculos M12 y M13 eran inervados por las neuronas CCAP durante el estadio larvario. Nuestros resultados indican que variaciones de la expresión de *opa* en las neuronas CCAP no tiene consecuencias sobre la correcta innervación de estos músculos. Asimismo, hemos podido identificar *de novo* los músculos inervados por las neuronas CCAP en el adulto.

Discusión Capítulo I

La inhibición de la expresión de *Antp* en el tejido presuntivo notum afecta a la correcta fusión entre los tallos del par de discos imaginales de ala. Este proceso no implica muerte celular, pero sí la alteración la vía de señalización JNK que produce cambios en el reordenamiento del citoesqueleto de actina en las células del tallo involucradas en la tracción y reconocimiento de cada disco.

No es la primera vez que una función de reorganización del citoesqueleto de actina se asocia a un gen Hox. De hecho, se sabe que los genes Hox son capaces de modular el mantenimiento de los bordes de compartimentos actuando sobre el citoesqueleto de actina. De manera interesante, el homólogo de *Antp* en nematodo *mab-5* es el responsable de la migración de los descendientes de los neuroblastos mediante la reorganización del citoesqueleto de actina en el frente de migración.

Un proceso morfogenético similar al del cierre del tórax adulto es el cierre dorsal del embrión. Sin embargo, se sabe que durante este estadio embrionario *Antp* únicamente se

requiere como gen de identidad segmental, lo que indica que Antp está implicado en movimientos de tejidos sólo durante los estadios post-embrionarios.

Sería interesante comprobar si esta nueva función de *Antp* en el cierre del tórax también está conservada en otros insectos. Previamente, se realizó una aproximación intentando alterar la expresión de *Antp* en mariposas, desafortunadamente, las limitaciones de las técnicas de inhibición de genes en esta especie no permitió llegar a una conclusión.

Discusión Capítulo II

Opa constituye un marcador ideal de las células CCAP debido a su expresión mas restringida en estas células, dado que los marcadores conocidos hasta ahora se expresan más ampliamente en el sistema nervioso central.

Opa mantiene vivas a las neuronas CCAP. Curiosamente, en el pez cebra se ha visto que la depleción de *Zic1* durante el desarrollo da lugar a apoptosis en el futuro diencefalo. Y la reducción de los niveles de expresión de *Zic2* tiene como consecuencia la muerte celular durante la diferenciación neuronal. Estos resultados señalan una conservación de la función de los genes *Zic* para prevenir la muerte de las células neuronales.

Opa constituye el primer regulador identificado de la expresión de Burs, el neuropéptido implicado en la ecdisis de los artrópodos. Sería interesante comprobar la expresión de *opa* en otras especies donde la posición de las células CCAP ya se conoce. Como ejemplo de la posible conservación funcional de Opa se sabe que en los coleópteros la inhibición de la función de *opa* en todo el cuerpo del animal da lugar a defectos en la metamorfosis.

En el estadio pupal de *Drosophila,* las motoneuronas CCAP cambian sus proyecciones axonales bajo control de la insulina. Aunque hemos visto que Opa no altera el comportamiento de los axones de las CCAP durante el estadio larvario, sería interesante comprobar si la arborización axonal de las neuronas CCAP adultas se afecta por la alteración de la expresión de *opa*.

Summary

Arthropods cuticle constitutes an external skeleton or exoskeleton. It has a protective function, such as support or defense against predator, but also has a physiologic role by preventing from desiccation or by sensing its environment among others. Arthropods suffer various molting throughout their lifetime. Their cuticle is highly rigid and has to be periodically shed to allow the animal to grow or to change its form. This process highly stereotyped, referred as ecdysis, is followed by a postecdysis period which permits the cuticle to become mature.

Drosophila melanogaster is a holometabolous insect. Its life cycle starts with the fecundated egg. At the end of the embryogenesis, the embryo hatches to give rise to a small size larva. Two molts are necessary to obtain the grown larva, which then stop moving. The cuticle of the larvae is then shed and used as a cocoon, called pupal case, where a complete metamorphosis (from the Greek *meta* “change” and *morphe* “form”) occurs. At the final ecdysis (eclosion), the adult fly emerges. During the post-eclosion behavioral sequence the newly formed and soft cuticle get tanned, that is to say get hardened and darken, and the wings extend.

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Abbreviations

20E 20-hydroxyecdysone
β-gal β-galactosidase
A Abdominal segment
Abd-A Abdominal-A
Abd-B Abdominal-B
Act Actin
ANT-C Antennapedia Complex
Antp Antennapedia
AP Antero-Posterior
APF After pupal formation
AS-C Achete-Scute Complex
bp base pair
Bsk Basket
Burs Bursicon
BX-C Bithorax Complex
°C degree Celsius
cAMP cyclic AMP
CB Central brain
Cbx Contrabithorax
CCAP Crustacean cardioactive peptide
CCAP-IN CCAP interneuron
CCAP-MN CCAP motorneuron
cDNA complementary DNA
Ci Cubitus interruptus
CNS Central nervous system
Da Dalton
Dac Dachshund
DC Dorsocentral
Dcr-2 Dicer-2
DDC Dopa decarboxylase
Dfd Deformed
Diap1 Death-associated inhibitor of apoptosis 1
DN Dominent negative
DNA Deoxyribonucleic acid
DOPA 3,4-dihydroxyphenylalanine

DP Disc proper
Dpp Decapentaplegic
DV Dorso-Ventral
EcR Ecdyzone receptor
En Engrailed
Ey Eyeless
Eyg Eyegone
FL Phalloidin
Flp Flipase
FRT Flp recombinase target
GFP Green flourescent protein
h hours
hh hedgehog
hs heat shock
JNK c-Jun N-terminal kinase
k kilo
L Instar larvae
lab labial
LTM Lateral tergosternal muscles
m meter
M muscle
min minute
MIP myoinhibitory peptide
mRNA messenger RNA
N Notch
NADA N-acetyl dopamine
NB Neuroblast
NBAD N-alanyl dopamine
Nub Nubbin
Odd Odd skipped
OL Optic lobe
Opa Odd paired
Pb Proboscipedia
Pburs Partner of bursicon
PE Peripodial epithelium
PELM pleural external longitudinal muscles
PITM pleural internal transverse muscles
PKA protein kinase A
pMad phosphorylated Mad
Pnr Pannier
PrDi Proximo-Distal
PS Parasegment
PTTH Prothoracicotropic hormone
Puc Puckred
RNA Ribonucleic acid
RNAi RNA interference
Rk Rickets
Rpr Reaper
RT Room temperature
S Segment
SE subesophageal
st stage
Scr Sex comb reduced
T Thoracic segment
TF Transcription factor
TH tyrosine hydroxylase
Tub Tubulin
UAS Upstream activator sequence
Ubi Ubiquois
Ubx Ultrabithorax
Ush U-shaped
VL Ventrolateral muscle
VM Ventral muscle
VNC Vental nerve cord
Wg Wingless
WT Wild type
y yellow
Y tyrosine
ZF Zinc finger
Zic Zinc finger in the Cerebellum
ZOC Zic-Opa conserved domain

Introduction

1 - *Drosophila melanogaster*, a holometabolous insect

Drosophila is a holometabolous insect, in other words it has a complete metamorphosis. The development of *Drosophila* has four well-defined stages: an embryonic phase, a larval period, a pupal stage and adulthood (**Figure 1A**). Its cuticle constitutes an external skeleton, called exoskeleton. To increase in size or to change shape, the animal must therefore periodically replace its cuticle by effectuating a molt; it has to get ride off the old one and replace it by a new one. Ecdysis permit the transition from the embryo to L1, L1 to L2, L2 to L3 and L3 to pupa where occurs the metamorphosis. Ecdysis is triggered by a pulse of ecdysone (**Figure 1B**), a steroid hormone synthesized from the dietary cholesterol in the prothoracic gland cells of the ring gland, a composite endocrine organ present nearby the Central Nervous System (CNS). Ecdysone synthesis and release are controlled by a brain-derived peptide called prothoracicotropic hormone, (PTTH) produced by two pairs of neurons located close to the midline of the central brain (CB), which innervate directly the prothoracic gland (reviewed by Ou and King-Jones, 2013). Once released into the hemolymph, ecdysone is converted in its targeted tissues to the active form 20-hydroxyecdysone (20E) that binds to its nuclear receptors (EcR).

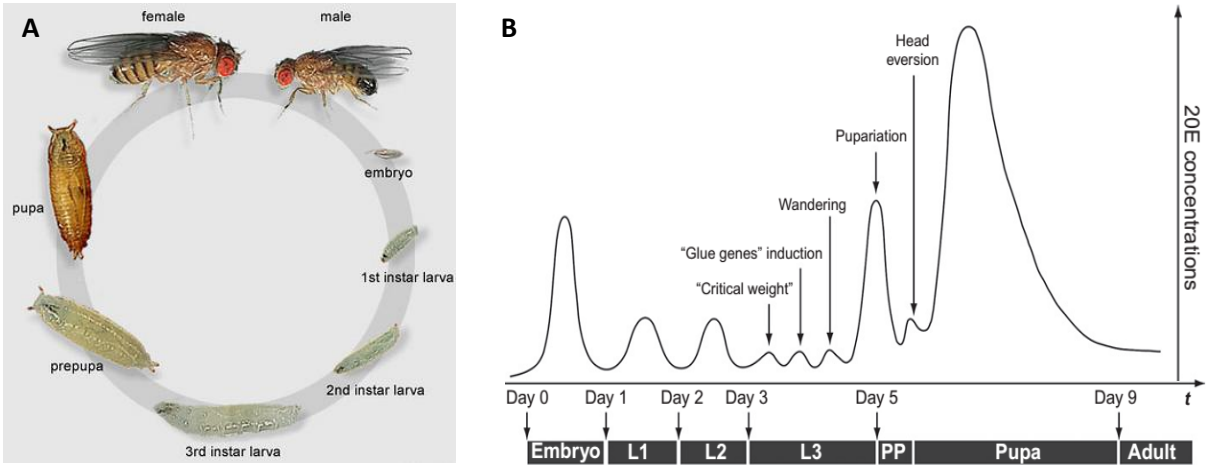


Figure 1. Presentation of the different *Drosophila* stages and their correlation with the pulse of active form of ecdysone (20E). (**A**) The life cycle is composed of four steps: an embryonic phase, a larval period, a pupal stage and adulthood. The development time from the zygote to the adult eclosion is temperature dependent. Indeed, it occurs in 9 days at 25°C, whereas it increases at 29°C and decreases at 17°C. At 25°C, embryogenesis is about one day. The mature embryo then hatches, resulting in a larva. Three larval steps or instars (L1, L2 and L3), separated by a molt, allow the larva to increase in size in a total of four days period. The larva, then, encapsulates in a cocoon called pupa to undergo a four-day-long metamorphosis; the larva cuticle disintegrates and is replaced by the adult structures. The pharate adult then emerges. Taken from Flymove. (**B**) Evolution of whole-body 20E concentration through *Drosophila* life cycle coincides with *Drosophila* molts. In addition, three minor pulses of 20E trigger physiological changes prior metamorphosis: critical weight checkpoint, immobilization of the mature larvae and stop feeding. The transition prepupa to pupa is marked by the eversion of the head. From Ou and King-Jones, 2013.

Adult cuticle derives from clusters of cells, called imaginal discs, which primordia arise in the embryo in a genetically well-defined position, at the level of the epidermis. Later during embryogenesis, they segregate from the ectodermal layer by invagination into the body (**Figure 2A**). Then, they grow during the larval stages (**Figure 2B**) but they do not contribute to the larval pattern. During the metamorphosis, while the larval cuticle disintegrates, the imaginal discs evaginate in a way that the central part of each imaginal disc gives rise to a functional limb (as a wing or a leg for example), whereas the periphery of these limbs fused with the adjacent ones to form the body wall (**Figure 2C**). Imaginal discs have their own size and shape and are named according to the appendage they form. The head derives from pairs of labial, clypeal and eye-antennal discs. The trunk derives from three pairs of leg discs, one pair humeral discs, one pair of wing discs, as well as one pair of haltere discs (structure involved in fly equilibrium). Finally, the most terminal parts of the fly, which correspond to the genitalia and anus, arise from a single genital disc.

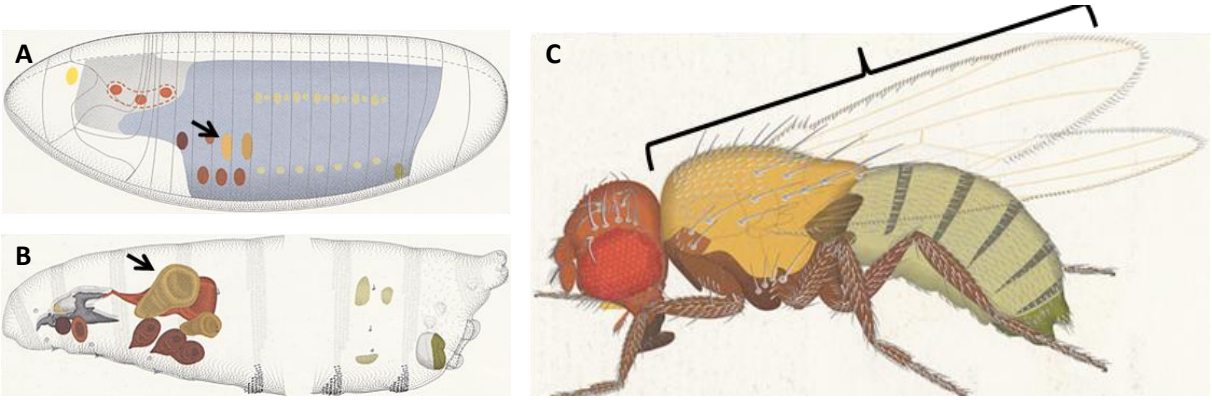


Figure 2. Origin of the adult cuticle: role of the imaginal discs. Localization of the different imaginal discs at the end of embryogenesis **(A)**, in a L3 larva **(B)** and their respective contribution of the adult cuticle **(C)**. The wing imaginal disc and the corresponding adult structure are pointed out by the arrows and the bracket. From Flybase.

2 - Metamorphosis and imaginal discs fusion

At the metamorphosis, the two wing imaginal discs everge and their respective Proximo-Dorsal part converge at the midline to fuse. This morphogenetic event is referred as thorax closure and interestingly shares an apparent similarity with the process of wound healing where, by a process of zippering, two-piece of tissue apart from each other join to form an uninterrupted structure.

The wing imaginal disc, like all imaginal discs, is a flat sac-like structure. It is composed of two polarized monolayer epithelia, which face to each other: the Disc Proper (DP) and the Peripodial Epithelium (PE) (**Figure 3A**). In L2, all the cells have the same cuboidal shape. Later, the shape of both epithelia evolves to adopt distinct features. Thus, in L3, the DP forms a columnar epithelium, whereas the PE corresponds to a squamous epithelium, because it is composed of thin and flat cells (McClure and Schubiger, 2005). The apical part of each epithelium faces the lumen; the basal part is oriented toward the basal lamina, which is in contact with the hemolymph.

Throughout embryogenesis and larval development, cells of the DP of the wing disc acquire positional information according to progressive establishment of the axes: Antero-Posterior (AP), Dorso-Ventral (DV) and Proximo-Distal (PrDi). This organization prefigures the polarization of the adult structure. During metamorphosis, the DP everts so the most central

cells will give rise to the most distal part of the adult structure. Conversely, the most lateral cells of the DP will form the most proximal part of the adult structures. By this way a two-dimensional larva epithelium gives rise to a three-dimensional adult structure. Three regions can be distinguished along the PrDi axis: the wing pouch, central region that gives rise to the proper wing, called wing blade; the ring-like domain around the wing pouch, named the hinge; and finally, the cells closest to the border are notum and pleura that give rise to the body wall **(Figure 3B**).

The destiny of cells that comprise the DP and the PE of the wing imaginal disc is radically different. Indeed, only the DP gives rise to the adult structure (see color code in **Figure 3A**

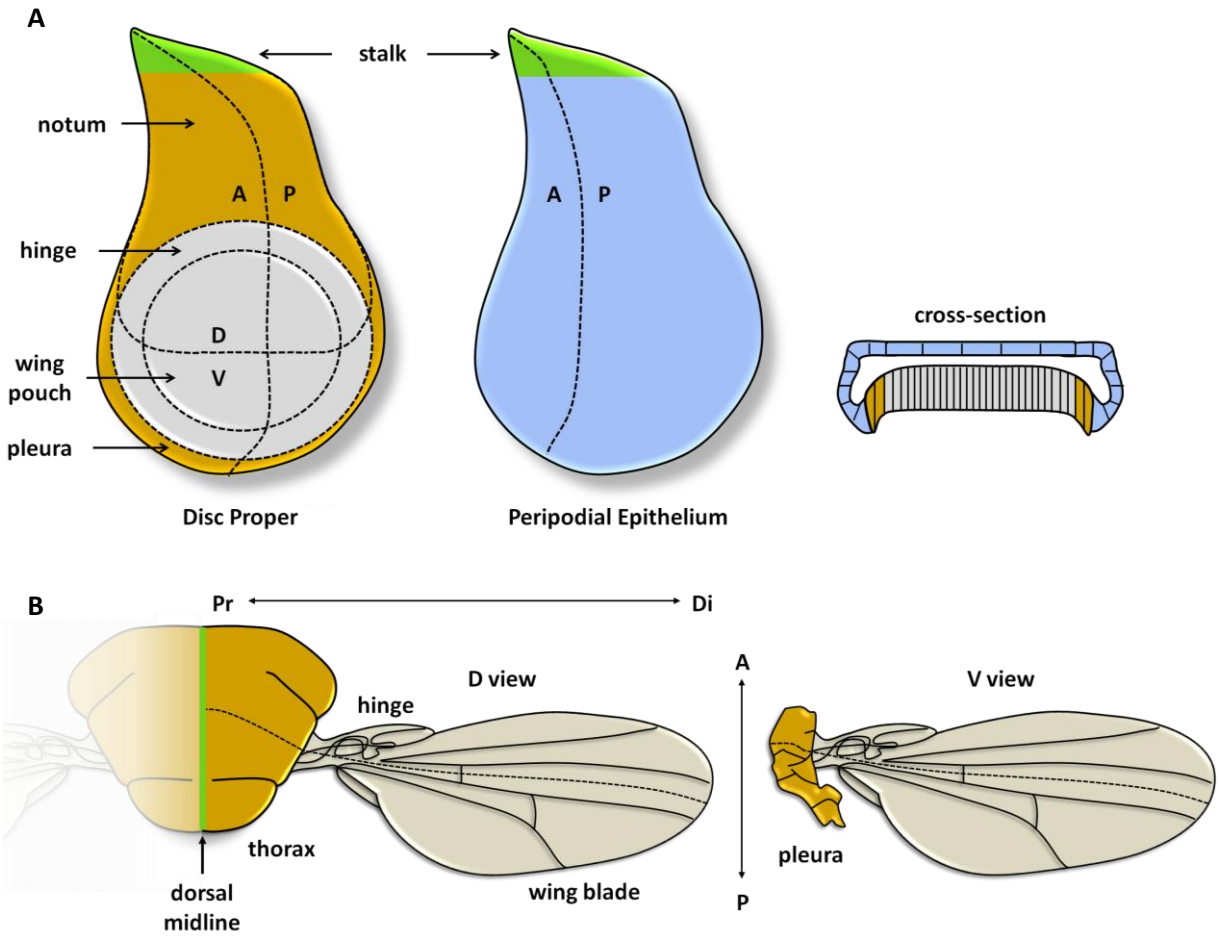


Figure 3. Fate map of the wing imaginal disc and its contribution to the adult structure. The wing imaginal disc **(A)** is composed of two layers, which face each other: a columnar epithelium, the Disc Proper and a squamous epithelium, the Peripodial Epithelium. The adult structure **(B)** derives mainly from the evagiation of the Disc Proper during the metamorphosis. The Peripodial Epithelium is poorly differentiated and does not give rise to a proper adult structure but it is crucial during the process of eversion of the Disc Proper and also during the fusion of the pair of everted discs at the level of the stalks to form a continuous adult thorax.

and **B**). Most of the PE is degraded during the metamorphosis. However, it has been demonstrated that, during the larval stage, signals emanating from the PE control the correct DP size and patterning (Gibson et al., 2002; McClure and Schubiger, 2005; Paul et al., 2013). Later during development, PE permits the DP to evert as an external appendage by cells contraction under the control of ecdysone signaling (Milner, 1977; Milner et al., 1984; Fristrom and Fristrom, 1993). Also, the stalk, corresponding to the most dorsal part of the imaginal disc which is attached to the body wall of the larva, helps the fusion of the couple of imaginal discs to form a continuous adult thorax (Usui and Simpson, 2000).

During the metamorphosis, at 5h after pupal formation (APF), cells of the stalk lose the apico-basal polarity leading to an epithelial-mesenchymal transition (Pastor-Pareja et al., 2004), actin cytoskeleton gets reshape and actin-based filopodia are observed at the leading edge (Martin-Blanco et al., 2000; Pastor-Pareja et al., 2004). These filopodia, using the larval epidermis as substrate, are believed to have a mechanic role during the thorax closure by pulling the couple of heminotum to the midline. They are also assumed to be implicated, once the stalks are at the midline, in cell-cell recognition, and finally in cell-cell fusion. Similar mechanism is used during Drosophila embryo dorsal closure or during nematode ventral closure (reviewed in Martin-Blanco and Knust, 2001). Notum fusion is a stereotyped process which describe a zippering movement: first, the most anterior cells meet at the midline, then the most posterior ones, and lastly cells of the middle get closer (Martin-Blanco et al., 2000; Usui and Simpson, 2000). No cell death is linked to this process (Martin-Blanco et al., 2000; Joza et al., 2008; Yoshioka et al., 2008).

Two signaling pathways have been identified as regulators of thorax closure. The first one is Decapentaplegic (Dpp) signaling, which is activated all along the AP compartment border of the wing disc. Indeed, alteration of Dpp signaling at different level of the pathway has been performed by different groups, which report Dpp signaling as having an important role in thorax patterning (Spencer et al., 1982; Morimura et al., 1996; Simin et al., 1998; Hudson et al., 1998; Chen et al., 1998). Interestingly, Dpp signaling has been also shown to regulate filopodia formation in the stalk (Martin-Blanco et al., 2000). The second signaling pathway involved in thorax closure in the c-Jun N-terminal kinase (JNK) signaling (**Figure 4A**). JNK signaling is specifically activated in the stalk and the PE (**Figure 4B** and not shown). Similarly, alteration of components of JNK signaling pathway lead to a non fused thorax (**Figure 4C**) (Zeitlinger et al., 1997; Takahashi et al., 1998; Agnes et al., 1999; Zeitlinger and Bohamann, 1999; Pastor-Pareja et al., 2004) which, like Dpp signaling, promote actin dynamics in the

stalk (Martin-Blanco et al., 2000). JNK signaling in the PE is also implicated in the evertion of the DP outside the body wall. Indeed, alteration of JNK signaling in the PE leads to a wing that remains as an internal structure into the adult fly (Agnes et al., 1999).

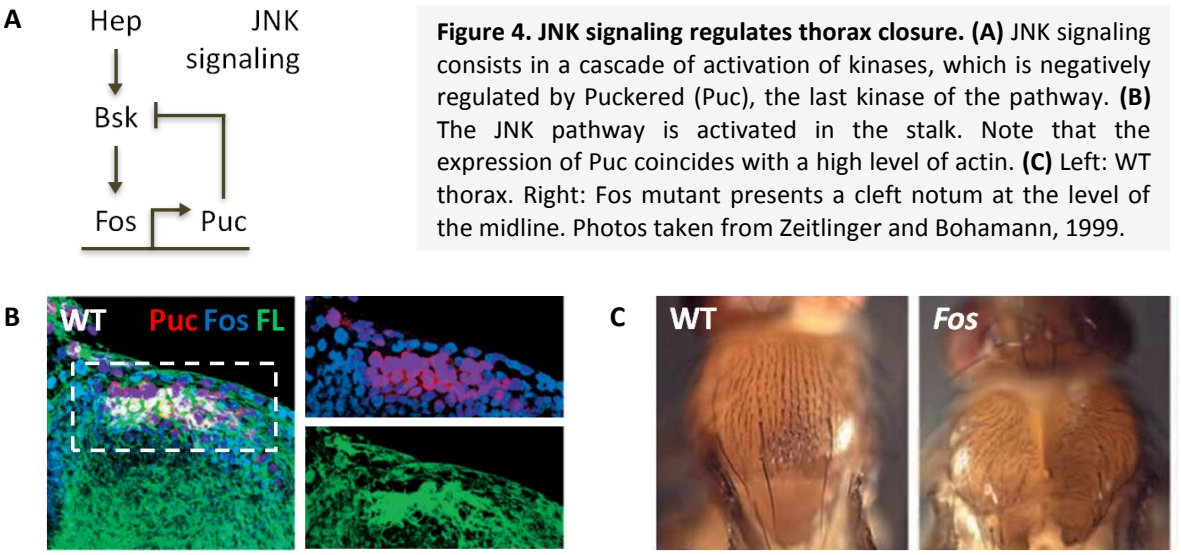


Figure 4. JNK signaling regulates thorax closure. (A) JNK signaling consists in a cascade of activation of kinases, which is negatively regulated by Puckered (Puc), the last kinase of the pathway. **(B)** The JNK pathway is activated in the stalk. Note that the expression of Puc coincides with a high level of actin. **(C)** Left: WT thorax. Right: Fos mutant presents a cleft notum at the level of the midline. Photos taken from Zeitlinger and Bohamann, 1999.

In *Drosophila*, a process analogous to the adult thorax closure is the dorsal closure of the embryo where cells of the leading edge of the epidermis, surrounding the amnioserosa, activate JNK signaling and emit filopodia allowing the embryo to close in a manner of two slide fasteners, head to head, gradually as the amnioserosa shrinks (Wood et al., 2002; reviewed in Harden, 2002). The mechanism of adult thorax closure or the embryonic dorsal closure is reminiscent of the logic of wound healing. Indeed, studies in the wing imaginal disc, larval epidermis and adult epidermis revealed that, after an injury, the tissue operates an event of zippering to close the cut by involving filopodia emission at the edge of the wound. Besides, cells of the border of the wound activate ectopically JNK signaling, and inhibition of JNK signaling after the injury impedes the healing (Ramet et al., 2002; Galko and Krasnow, 2004; Bosch et al., 2005). Similar results have been reported after cell death-induced lesion (Bergantinos et al., 2010; reviewed in Repiso et al., 2011). For all these reasons, thorax closure is a good model to study the response induced after a cut. Besides, a recent screening has been performed altering thorax closure to identify wound healing regulators (Alvarez-Fernandez et al., 2015).

3 - Adult ecdysis: the CCAP neurons and the abdominal muscles

Later during the pupal ecdysis, at 12h APF, the pupa presents similar morphology to the adult. The different appendages have fused with the sibling ones and they have completely everted, and the head, first inside the thorax, is pushed out to the anterior part. Muscle contractions lead to a pulse of hemolymph, which generate pressure inside the body and forces the externalization of these different body parts. A similar mechanism is implicated afterward during the adult postecdysis when the newly eclosed adult expands its wing and stretches its thorax. This event is highly stereotyped and occurs during the first 30 min of the adulthood (Park et al., 2003).

Also, in a 3h time window after eclosion, the newly formed and soft cuticle gets tan by incorporation of phenolic compounds. Tanning includes cuticle hardening (also called sclerotization) and pigmentation (darkening or melanization) to prevent desiccation (**Figure 5A**). It involves first the amino acid tyrosine (Y), which is hydroxylated by tyrosine hydroxylase (TH) to 3,4-dihydroxyphenylalanine (DOPA) and then decarboxylated by dopa decarboxylase (DDC) to dopamine. For sclerotization, dopamine N-acetyltransferase acylates dopamin to N-acetyl dopamine (NADA) and N-alanyl dopamine (NBAD). NADA and NBAD are secreted from the epidermis into the cuticle where they are oxidized and cross-linked with chitin, a modified polysaccharide characteristic of arthropod exoskeleton, making it to be hardened and hydrophobic. For cuticle melanization, dopamine is also converted to insoluble melanin via 5,6-dihydroxyindole. Melanin is linked to granular proteins or is distributed throughout the cuticular matrix to give the cuticle a pigmented aspect (reviewed by Dong and Song, 2012).

The crustacean cardioactive peptide (CCAP) neurons are neurosecretory cells, identified in crustaceans and insects, and are important regulators of the postecdysis process. In the fly, 50 CCAP are found in the CNS (in L3) and present a segmented pattern. Along the VNC, all the hemisegment (at the exception of the most anterior and posterior ones) have two CCAP. CCAP neurons synthesize the hormone Bursicon (Burs; derived from the Greek word *bursikos*, pertaining to tanning) (**Figure 5B**; Fraenkel and Hsiao, 1962; Cottrell, 1962). Burs is a 30kDa heterodimeric cystine knot protein formed by Burs and Partner of bursicon (Pburs) (**Figure 5A**), also called αburs and βburs respectively (Luo et al., 2005; Mendive et al., 2005). At the onset of the metamorphosis, Burs and Pburs are released to the hemolymph and act

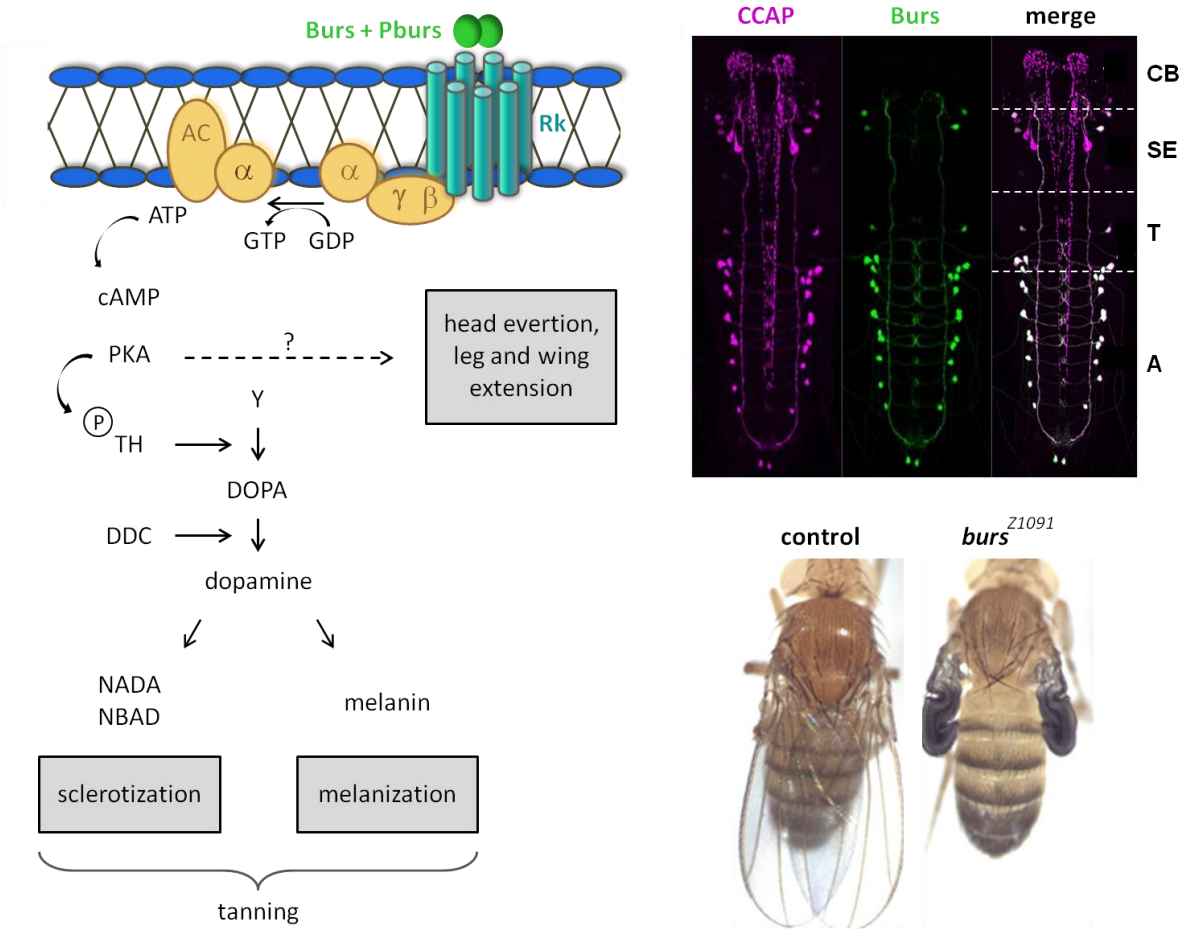


Figure 5. Role of the neuropeptide Bursicon during *Drosophila* adult post-ecdysis. (**A**) Schematic representation of the Bursicon signaling. The pathway is triggered upon the binding of the heterodimer Burs + Pburs on the ubiquitous receptor Rk that leads to an increase of cAMP. cAMP activates PKA which phosphorylates TH, which catalyzed the reaction Y to DOPA. DOPA is the precursor of the dopamine which is either converted in NADA and NBAD, metabolites implicated in the sclerotization of the cuticle, or in melanin involved in cuticle pigmentation. As indicated in the scheme, it is not known how PKA controls head eversion or legs and wings extension. Scheme adapted from An et al., 2014. (**B**) Double labeling of the neuropeptide CCAP to mark the CCAP neurons and the neuropeptide Burs. *Burs* is expressed in most of the CCAP neurons in a CNS of a mature L3. CB: central brain; SE: subesophageal; T: thoracic and A: abdominal segments. Photos from Honegger et al., 2008. (**C**) Comparison of the phenotype of a 3h control adult fly and a hypomorphic individual for *Burs* at the same age showing the characteristic defects of the adult post-ecdysis sequence: lack of wing expansion, lack of thorax extension, and cuticle tanning defect. Photos from Dewey et al., 2004.

upon binding to the G protein-couple receptor Rickets (Rk), which leads to a transient increase of the second messenger cyclic AMP (cAMP) in the target tissues which activates the protein kinase A (PKA) (Luo et al., 2005; Mendive et al., 2005). PKA in turn stimulates the phosphorylation of TH, which leads to the hydroxylation of tyrosine to DOPA, the precursor of dopamine involved in the process of tanning (Davis et al., 2007). However, it remains unknown how-high level cAMP regulates the hemolymph flow needed for appendages eversion or extension (**Figure 5A**).

Genetic ablation of CCAP neurons by ectopically expressing the proapoptotic gene *reaper* (*rpr*) under control of a *CCAP-Gal4* driver leads mainly to a pupal lethality with defects in the extension of the wings, the legs and the eversion of the head. Few individuals, however, make a complete ecdysis and eclose but fail to extend their wings and thorax and present defect in the tanning (Park et al., 2003). Similar defects are observed in mutant alleles for *Burs* (**Figure 5C**; Dewey et al., 2004), *Pburs* (Lahr et al., 2012) or for *rk* (Baker and Truman, 2002; Loveall and Deitcher, 2010).

In the Ventral Nerve Cord (VNC), each hemisegment contains one CCAP interneuron and, depending of the hemisegment, one CCAP motoneuron is present nearby. Both cells are not siblings but belong to the lineage of the Neuroblast (NB) 3-5. CCAP neurons are generated early during development, between embryonic stage (st) 9 and 11, during the two first rounds of division of the NB 3-5 (**Figure 6A**; Moris-Sanz et al., 2014). The hormonal differentiation appears, for most of them, in embryonic st 17 (Moris-Sanz et al., 2014) and then much later, at the entrance of pupal stage, in a subset of CCAP cells localized in the

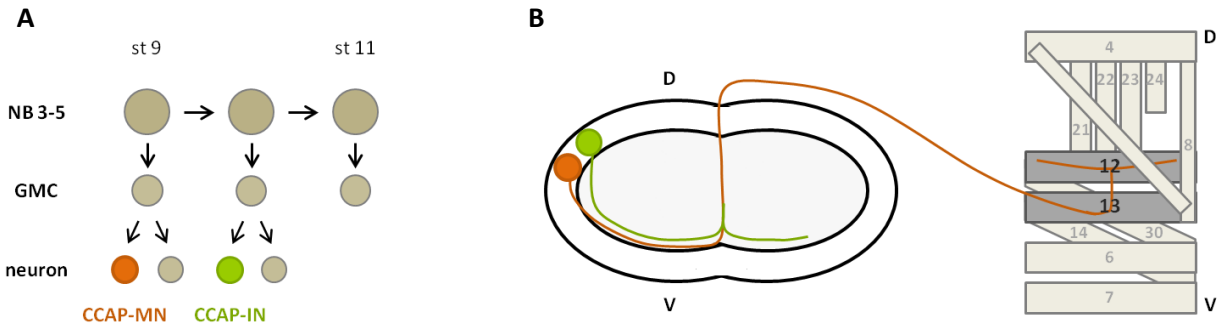


Figure 6. Lineage of the NB 3-5 and axonal projections of the CCAP motoneuron (CCAP-MN) and CCAP interneuron (CCAP-IN). (**A**) The NB 3-5 delaminates from the neurectoderm in each hemisegment at stage (st) 9. It divides once to generate the CCAP-MN, and a second time to give rise to the CCAP-IN. Modified from Moris-Sanz et al., 2014. (**B**) In each hemisegment the CCAP-MN and -IN are close to each other. As seen in a cross-section at the level of the VNC, they have a dorso-lateral position in the VNC. The axon of the CCAP-MN crosses the midline, and exits the VNC dorsally to innervate the muscles M12 and M13 present on the ventral side of the larva. The axon of the CCAP-IN crosses the midline and stay in the CNS. Modified from Karsai et al., 2013.

posterior VNC (Veverytsa and Allan, 2012).

Prior to metamorphosis, Burs starts to be released along the CCAP axons and its expression completely disappears in 24h old adult flies (Lee et al., 2013). The CCAP neurons get progressively eliminated by apoptosis in the adulthood; the last ones are detected 4 days after eclosion (Peterson et al., 2002; Lee et al., 2013). The nature of the CCAP neurons, motoneuron or interneuron, has been established thanks to the observation of the axon projection (Peabody et al., 2008; Karsai et al., 2013). In the case of the axons of CCAP motoneurons, all of them first cross the VNC midline and innerve the larva muscles M12 and M13 (also called VL1 and VL2 respectively) (Hodge et al., 2005; Karsai et al., 2013). In the case of the CCAP interneurons, their axons also cross the midline but their axons remain within the CNS (Karsai et al., 2013), establishing still unknown neuron contacts (**Figure 6B**).

CCAP neurons are highly specialized peptidergic cells, which in addition to *Burs* express two other neuropeptides. The first one is the CCAP neuropeptide (**Figure 5B**), first identified in the sphinx moth *Manduca sexta* and the shore crab *Carcinus maenas* as a modulator of heart contraction (Tublitz and Evans, 1986; Stangier et al., 1987) and later in the fly (Nichols et al., 1999; Dulcis and Levine, 2003; Dulcis et al., 2005). It has been suggested that the cardioacceletory property of neuropeptide CCAP in *Drosophila* could favor the hemolymph flux and therefore a correct distribution of Burs to its target tissues (Ewer, 2005). This hypothesis seems to be unlikely given that the mutant flies bearing *CCAP*-null allele are viable and do not present a particular phenotype (Lahr et al., 2012). However, in *M. sexta* CCAP is known to control hemolymph circulation required for wing inflation (Tublitz, 1989). The other neuropeptide expressed in the CCAP neurons is the myoinhibitory peptide (MIP, also known as AstB). MIP neuropeptide inhibits spontaneous muscle contractions of the gut and oviduct of the locust *Locusta migratoria* and *M. sexta* (Schoofs et al., 1991; Blackburn et al., 1995). In *Drosophila*, MIP is believed to be involved in the clock system (Kolodziejczyk and Nassel, 2011), and its implication in the insect adult ecdysis is not so clear (Nassel and Winther, 2010).

As previously mentioned, organs externalization or wings extension is mediated by abdominal contractions that generate a hemolymph pulse. While most of the adult muscles are formed during metamorphosis, the abdominal muscles required for eclosion remain from the larva and degenerate gradually during the first hours after eclosion, once they have accomplished their function (at 12h they are completely degraded) (**Figure 7**; Kimura and

Truman, 1990). No adult muscle has been described to be innervated by the CCAP yet. Therefore, these doomed muscles constitute ideal candidates to be innervated by the CCAP motoneurons surrounding the eclosion.

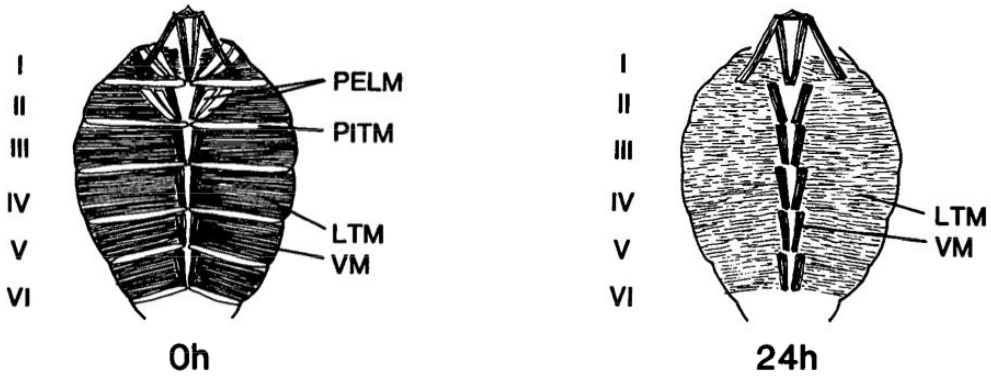


Figure 7. Evolution of the abdominal muscular system at eclosion and at 24h upon eclosion. Left: ventral abdominal musculature of an adult at 0h. Right: ventral abdominal musculature of an adult at 24h. PELM: pleural external longitudinal muscles; PITM: pleural internal transverse muscles; LTM: lateral tergosternal muscles; VM: ventral muscles. Modified from Kimura and Truman, 1990.

4 - The transcription factor Opa, a Zic family member

Part of this Thesis aims to reveal a new role of the *Drosophila* homolog *Zinc finger in the Cerebellum* (*Zic*) in the ecdysis process, which is why we present here the known functions of the *Zic* genes during development.

Zic family members are transcription factors (TF) with five characteristic tandem repeats of C2H2-type zinc finger (ZF) motifs. They belong to the GLI/GLIS/ZIC ZF superfamily proteins. Zic homologs have been identified in most of metazoans and are thought to have derived from a common ancestral gene (reviewed in Aruga et al., 2006; Layden et al., 2010). *Drosophila* possesses a single *Zic* homolog, *odd-paired* (*opa*), whereas both mice and humans have 5 homologs (*Zic1-5*). Throughout species, the ZF domain is highly conserved. Also, two smallest regions are conserved, although in a minor scale. The first one, just upstream the ZF domain, is the ZF-NC. This sequence is present in arthropods and in vertebrates but it is absent in the single nematode homologue, *Ref-2*. The functional role of this conserved sequence has not been studied yet. The second one, located close to the N terminal part, is the ZOC (Zic-Opa conserved) domain, conserved between the fly *opa* and

the mouse *Zic1-3*. However, the mouse *Zic4* and *Zic5* as well as the nematode *Ref-2* lack this sequence. The ZOC domain has been shown to be necessary for the transcriptional activation capacity of the mouse Zic2 protein (Mizugishi et al, 2004).

Zic members are involved in a wide spectrum of developmental processes. *Ref-2* has a role in nematode vulva development. *opa* is implicated in the correct fly embryo segmentation as a pair-rule gene in *Drosophila* (Benedyk, et al., 1994). It is also important for embryo midgut constriction (Cimbora and Sakonju, 1995) and adult head morphogenesis (Lee et al., 2007). In mice, each of the 5 homologs has its own role; they are not functionally redundant. *Zic1* is important for proper skeletal patterning while *Zic2* is implicated in forebrain formation. Mutation in *Zic2* results in holoprosencephaly, the most common congenital malformation of the forebrain. *Zic3* is crucial for left-right axis establishment and *Zic4* is essential for normal cerebellar foliation. Mutation in *Zic4* is associated to the Dandy-Walker malformation, the most common congenital malformation of the cerebellum. Finally, *Zic5* is fundamental for facial bone formation. Similar role has been reported for human homologs, putting thus the *Zic* family genes as central key factors for correct development (reviewed in Grinberg and Millen, 2005).

5 - Hox genes

Part of this Thesis is dedicated to the study of the requirement of the Hox gene *Antennapedia* (*Antp*) during thorax closure. Therefore, in the following part, we introduce the Hox genes family and their known role in *Drosophila*.

In *Drosophila*, like in all the arthropods, the body plan is organized in metameric units. The segmentation step occurs during the embryogenesis stage, which sequentially subdivide the embryo into progressively smaller and smaller units. Each of theses metameres receives later identity cues, provided by the Hox genes. This embryonic organization in subunits with their own identity is conserved in the larva and in the adult fly (reviewed in Hughes and Kaufman, 2002).

As a long germ band insect, all the segments of the fly are specified simultaneously. Segmentation starts during oogenesis with asymmetric distribution of maternal messenger

RNA (mRNA) in either the anterior or posterior pole of the egg. After fertilization, these mRNA are translated and the resulting proteins form long-range concentration gradients into the syncytial egg. Maternal products activate the zygotic gap genes in wide but well-defined domains along the AP axis. These genes encode TF that activate expression of the pair-rule genes. Each of these genes is expressed in 7 stripes in a periodic manner with a two-stripe periodicity. Thus, pair-rule genes expression defines 14 subdomains, called parasegments (PS). These genes encode for TF that activate the expression of segment polarity genes. These last ones are expressed in 14 stripes, one in the same position in each PS and encode not only for TF, but also for receptors and ligands. At this step, each PS is subdivided into 2 compartments. Each compartment maintains a lineage restriction barrier. Compartments received their identity by the expression, or lack of expression of selector genes. The segment polarity gene *engrailed* (*en*), which encodes a TF, acts as selector gene by conferring Posterior (P) compartment identity. Anterior (A) compartment is characterized by the lack of expression of *en*. An A and a P compartment form one segment, whereas one PS (or metamere) comprise a P compartment and the A compartment of the following segment (**Figure 8**) (reviewed in Peel, 2004; Patel and Liu, 2009).

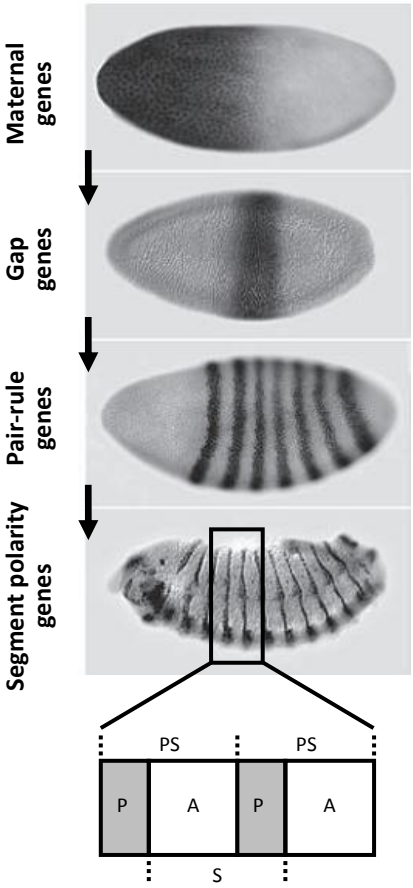


Figure 8. Embryonic segmentation. Sequential expression of maternal, gap, pair-rule, and segment polarity genes serves to subdivide the egg into finer and finer regions. For each step one example of gene pattern is shown. PS: parasegment; S: segment; A: anterior compartment; P : posterior compartment. Modified from Patel et Liu, 2009.

The AP axis polarization of the body is conferred by the highly conserved Hox genes (reviewed Durston et al., 2012). The gap, pair-rule and segment polarity genes control Hox genes expression. In addition, in the embryo, a given posterior Hox gene represses the expression of the one anterior to it; this mechanism is called posterior suppression (Struhl, 1983; Busturia and Morata, 1988). The maintenance of their expression is controlled by the chromatin remodeling proteins Polycomb and Trithorax (Duncan, 1982; Busturia and

Morata, 1988). Also, some of Hox genes are known to regulate their own expression (Bergson and McGinnis, 1990; Chouinard and Kaufman, 1991; Irvine, 1993). Hox genes encode for TF, with a characteristic helix-turn-helix DNA-binding domain of 60 aminoacids, named the homeodomain, which binds DNA sequences rich in A and T nucleotides and can act as activator or repressor of transcription. They control the development of the ectoderm, the mesoderm and the endoderm by regulating genes involved in a wide spectrum of cellular and morphogenetic functions (reviewed in Hueber and Lohmann, 2008; Sanchez-Herrero, 2013).

Hox genes are organized into two clusters on the right arm of the third chromosome. The first one is the Antennapedia Complex (ANT-C) which is composed of *Labial* (*Lab*), *Proboscipedia* (*Pb*), *Deformed* (*Dfd*), *Sex comb reduced* (*Scr*) and *Antennapedia* (*Antp*) genes. The second one, found 10 Mbp farther, is the Bithorax Complex (BX-C), which is composed of *Ultrabitrorax* (*Ubx*), *Abdominal-A* (*Abd-A*) and *Abdominal-B* (*Abd-B*) genes (**Figure 9A**). Their expression limits coincide with PS boundary. This segmentation process, followed by Hox gene expression along the AP axis, permits the embryo to be divided in three regions, also known as tagmata: the head, thorax, and abdomen. The head is composed of six segments (antennal, ocular, intercalary, mandibular, maxillary, and labial), the thorax of three segments (T1-T3) and the abdomen of eleven segments (A1-A11) (**Figure 9B**). This organization is conserved in the adult (**Figure 9C**).

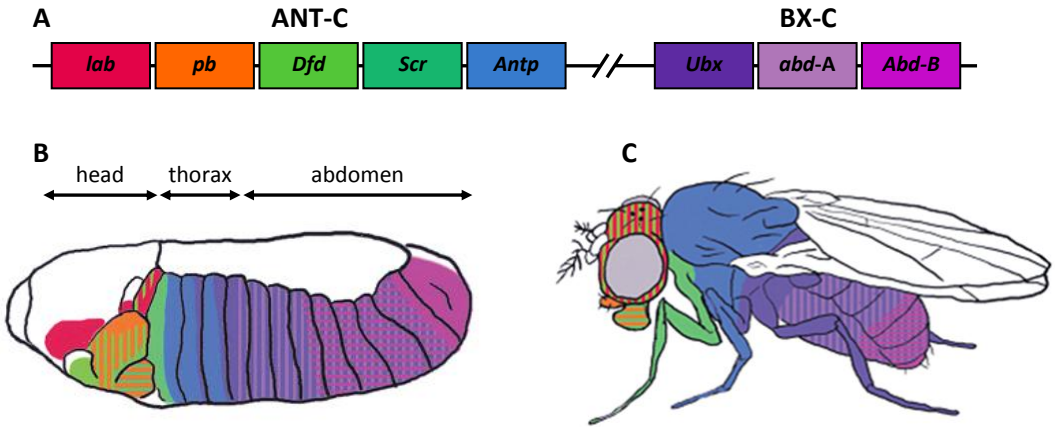


Figure 9. Hox genes expression in the ectoderm. (A) Hox genes are distributed into two complexes, ANT-C and BX-C, on the III chromosome. **(B)** Hox genes expression pattern along the AP axis of the late embryo. Their expression domain is maintained in adult stage **(C)**. Modified from Hughes and Kaufman, 2002.

Hox genes are determinant of identity. They received their name because of the phenotype produced when missexpressed, called homeotic transformation, name derived from the word homeosis (Bateson, 1894). Homeotic transformation refers to the genetic reprogramming of the fate of one organ to another. This is only possible between organs that share similar or homologous developmental cues (ventral appendages: antenna, leg and genitalia *versus* dorsal appendages: wing and haltere). Homeotic transformations reveal the ability of Hox genes to control the morphologic diversity of the fly body plan and also mutations in these genes have been able to introduce body plan changes through evolution.

What differentiates the identity between two homologue structures is the expression of a Hox gene, a combination of Hox genes or the lack of Hox gene expression. For example the Hox gene *Ultrabithorax* (*Ubx*) discriminates between wing and haltere fate. Ubx is expressed in the haltere imaginal disc whereas it is absent from the wing disc. Ubx acts as a selector of haltere identity by repressing the wing-patterning genes that are required for the growth and features of the wing (Weatherbee et al., 1998; de Navas et al., 2006). Absence of *Ubx* function in the haltere disc results in transformation to wing tissue (Morata and Garcia-Bellido, 1976; Lewis, 1978). On the contrary, mutations that cause ectopic expression of *Ubx* (e.g *Contrabithorax* (*Cbx*)) in the wing disc (Cabrera et al., 1985; White and Akam, 1985) transform it into haltere. Also, the expression of the Hox gene *Antennapedia* (*Antp*) in the T2 leg but not in the antenna establishes the difference between the antenna and the second thoracic (T2) leg. Ectopic expression of *Antp* in the antenna leads to an antenna-to-leg transformation (Postlethwait and Schneiderman, 1971) and conversely inhibition of *Antp* expression in the T2 leg induces a leg-to-antenna transformation (Struhl, 1981; Emerald and Cohen, 2004); hence the name of the gene, literally antenna-foot.

Objectives

1 - To define the expression profile of the Hox gene *Antennapedia* (*Antp*) in the wing imaginal disc and to identify the signaling pathway(s) under Antp control during adult thorax closure.

2 - To understand how Odd paired (Opa) could control *Drosophila* ecdysis by establishing a functional correlation between CCAP neurons, required for adult ecdysis, and the Opa expressing neurons.

Material and methods

1 - Stock of flies used

Flies manipulation has been carried out according to Ashburner, 1989. Crosses have been set up in tubes containing medium of yeast, cornmeal, sugar, agar and water, at 25°C, room temperature (RT) or 17 °C, according the needs.

• Driver lines: *pnr-Gal4*, *nub-Gal4, odd-Gal4* (Calleja et al., 1996), *CCAP-Gal4* (Park et al., 2003), *AntpP1-Gal4* (Gehring), *386Y-Gal4* (Taghert et al., 2001), *36Y-Gal4* (Min), *elav-Gal4* (BDSC #25750), *77B01-Gal4*, *77B05-Gal4* and *77B12-Gal4* (BDSC #46977, #46979, #41307 respectively)

• Flip-out lines: *hs-flp*; *act > y+ > Gal4*, *UAS-GFP* (Pignoni and Zipursky, 1997)*; hs-flp*; *tub>GFP>Gal4* (Zecca and Struhl, 2002).

• UAS lines: *UAS-Ubx* (Castelli-Gair et al., 1994), *UAS-Antp* (BDSC# 7301), *UAS-puc* (Martin-Blanco et al., 1998), *UAS-bsk*^{DN} (Adachi-Yamada et al., 1999), *UAS-Diap1*, *UAS-p35*, *UAS-Dcr-2* and *UAS-GFP* (BDSC; to visualize axons we used BDSC #52261), *UAS-Zic2* from *Xenopus laevis* (generated in our lab).

• RNAi lines: *RNAi-Antp* (from VDRC #101774 (KK) on the II chromosome, here called *RNAi-Antp^{KK}* and from BDSC #27675 (Valium 10) on the III chromosome, here called *RNAi-Antp^{V10}*; *RNAi-opa* (from VDRC #51292 (GD) on the X chromosome, here referred as *RNAi-opa^{X}*, #101531 (KK) on the II chromosome, here called *RNAi-opa^{II}* and from BDSC #34706 (Valium 20) on the III chromosome, here named *RNAi-opa^{III}*).

• Mutant lines: *opa^{7}* (Benedyk et al., 1994), *Ubx^{1}* (Morata and Kerridge, 1981).

• FRT lines: *hs-flp; FRT82B Ubi-GFP* (BDSC).

• Reporter lines: *puc^{E69}-lacZ* (Martin-Blanco et al., 1998), *opa-lacZ* (Tix and al., 1997; its localization is described in Beckervordersandforth, 2007), *odd-lacZ* (Rauskolb and Irvine, 1999), *HhDsRed* (Akimoto et al., 2005), *dpp-lacZ$^{BS3.0}$* (Blackman et al, 1991).

• Other lines: *tub-Gal80^{ts}* (BDSC). Oregon fly strain is used as WT.

2 - Construction of *UAS-opa*

opa cDNA was amplified by PCR from LD30441 clone (Berkeley Drosophila Genome Project Gold cDNA; Drosophila Genomics Resource Center) using the sense primer: 5' CACCATGATGATGAACGCCTTCATT 3' and the anti-sense primer: 5' ATACGCCGTCGCTGCGCC 3', cloned in the entry vector *pENTR/D-TOPO* (Gateway system; Invitrogen) and introduced by recombination in the destination vector *pTWH*.

3 - Techniques of genes missexpression

• The UAS/Gal4 system

To express ectopically a cDNA or an RNAi in a specific domain, we used the UAS/Gal4 yeast system (Brand and Perrimon, 1993). The yeast Gal4 protein is under the control of a chosen promoter (e.g. X promoter). The construct X promoter + Gal4 is called a driver. Once expressed, according to the spatiotemporal pattern of the promoter, the Gal4, a

transcription trans-activator, recognizes its specific DNA binding site, called UAS (Upstream Activating Sequence), inserted upstream of the transgene of interest (**Figure 10**).

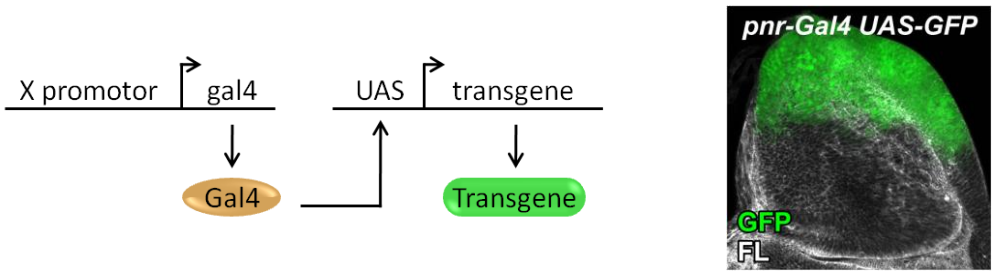


Figure 10. The UAS/Gal4 system. Left: schematic representation of the UAS/Gal4 system. Right: illustration of the UAS/Gal4 system with an example: *pannier (pnr)-Gal4 UAS-GFP* in L3. Here, the transgene *UAS-GFP* is under the control of the *pnr* promoter.

To control the temporal expression of a driver, we took advantage of the thermosensitive Gal80 (Gal80ts) protein. This protein is under control of the *tubulin* (*tub*) ubiquitous promoter. In permissive temperature conditions (17°C), the Gal80ts binds the Gal4, impeding this last one to activate the transgene. On the contrary, in restrictive temperature conditions (29°C), the Gal80ts protein is inactive, thus leading the Gal4 protein able to activate the transgene.

• The FRT/flp system

As the mutations employed in this study are homozygous embryonic lethal, we induced random mutant homozygous mitotic recombinant clones, lack of function clones, in a heterozygote background, using the FRT/flp (Flipase Recombination Target/flipase) yeast system (Xu and Rubin, 1993). The flp, a site-specific recombinase, is under the control of a *heat shock* (*hs*) promoter (activated at 37°C). Once expressed in a cell, the flp induces the recombination of two homologous chromosomes during the mitosis by recognizing a specific sequence called FRT. One of the chromosomes contains the mutant allele, whereas the other one has the wild-type allele and contains a ubiquitous GFP (Ubi-GFP). Thus, after mitosis, one of the daughter cell is homozygous mutant and the other, the twin, is wild type having twice GFP copies than the heterozygote cells. These two cells will divide producing a clone (or group of daughter cells) loss-of-function for the mutation and a wild type clone (**Figure 11**).

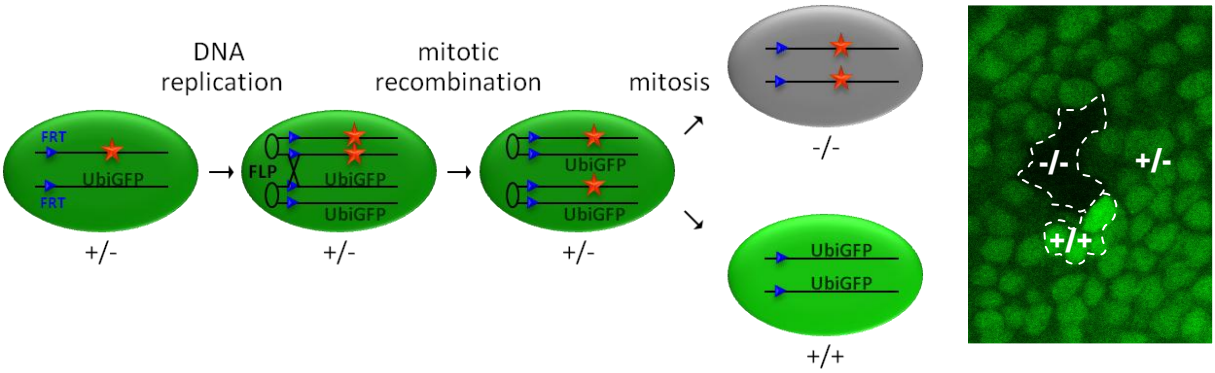


Figure 11. The FRT/flp system.

• The flip-out technique

The flip-out technique (Struhl and Basler, 1993) permits to express ectopically a cDNA or an RNAi in a random manner and requires the FRT/flp and UAS/Gal4 systems. The flp is under the control of a *hs* promoter. Once activated in a cell, the flp recombines two FRT sites, localized on the same chromosome and having the same orientation. The cis recombination leads to the deletion of the sequence found between these two FRT sites, allowing the ubiquitous promoter (*act* or *tub*), localized before the first FRT site, to be closer to the Gal4 trans-activator, present after the second one. Thus, the Gal4 expression, henceforth under control of the constitutive promoter, will bind to the UAS inserted up-stream the transgene

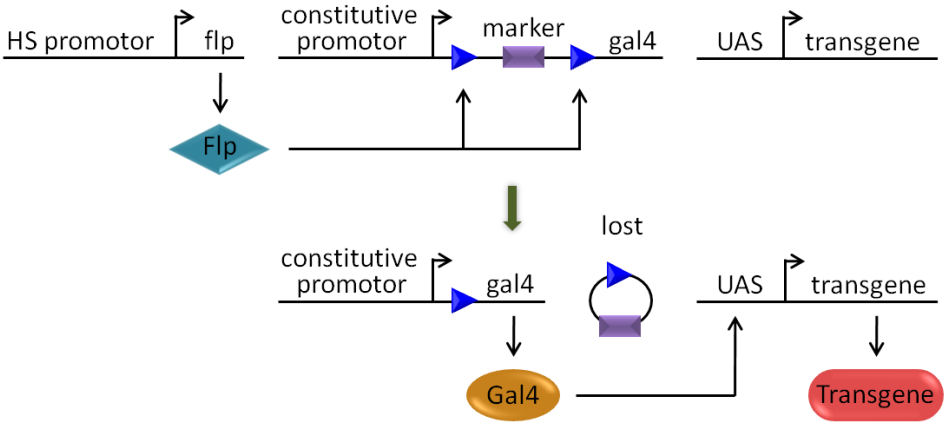


Figure 12. The flip-out system.

of interest (**Figure 12**). Cells could be marked positively via an *UAS-GFP* or conversely by the lack of GFP.

4 - Immunohistochemistry

We detect the presence of proteins by incubation of specific antibodies (primary antibodies) which in turn are revealed by secondary antibodies against the primary. The method of fixation depends on the tissue of interest. Imaginal discs or CNS of larvae, are dissected in phosphate-buffered saline (PBS) 1X and fixed during 30 min in 4% paraformaldehyde (PAF) in PBS at RT. Fillet preparation of L3 larvae and adults are directly dissected in 4% PAF and let them fixed for 1h-1h30. Pharate thoraxes are fixed in 4% PAF at 4°C overnight. Tissues are then washed 3 times for 20 minutes each in PBT: PBS + 0,1% Triton 100X. We saturate the unspecific sites by adding to the tissues 1% PAT (PBS, 0,1% triton, 1% Bovine Serum Albumin (BSA)) during 1h at RT. Then the primary antibody is added with the appropriate concentration in 1% PAT. The reaction is incubated overnight at 4°C. Later, after removing the first antibody, the dissected tissues are washed 3 times for 20 min each in PBT. The secondary antibody conjugated to a fluorochrome is then added. The reactions take place in the dark during 1h45 at RT. The samples are then washed 3 times of 20 min each in PBS. To finish, tissues are mounted in Vectashield medium (Vector Laboratories) between a slide and a cover slip.

First antibodies used: rabbit anti-β-gal 1:1000 (Jackson Laboratories), mouse anti-Wg 1:50, mouse anti-Dac 1:50, mouse anti-Ubx 1:50, mouse anti-Antp 1:100 and rat anti-Ci 1:5 (Iowa Hybridoma bank), ginea-pig anti-Eyg 1:200 (Aldaz et al., 2003), rabbit anti-Burs 1:2000 (Peabody et al., 2008).

Secondary antibodies used: goat anti-mouse o anti-rabbit conjugated with FITC, TRITC or Cy5 1:200, according to the first antibody used (Jackson Laboratories).

Phalloidin (FL)-TRITC 1:2000 (Sigma) was used to mark actin filaments and it was added together with the secondary antibodies.

5 - Images acquisition and processing

Images of fluorescent tissues were taken with a Zeiss Laser Scanning Confocal Spectral LSM710 with Axiolmager M2 upright microscope. Images of adult wings and mouth hooks of larvae were taken with a Zeiss Axioskop upright microscope coupled with a monochrome ccd camera. Images of adult flies and pupae were taken with a Leica M205 FA microscope coupled with a DFC 550 camera. Images were processed with Adobe Photoshop CS7 and ImageJ 1.46.

6 - Statistical analysis

Statistical analysis was performed with Student's t-tests using GraphPad Prism 5.0a. For all analysis the SEM (standard error of the mean) is shown.

Chapter I

The Hox gene *Antennapedia* regulates actin cytoskeleton for *Drosophila* adult thorax closure

Results

1 - Antp is expressed in the notum

We start the first Chapter of this Thesis by describing the expression pattern of the homeotic gene *Antennapedia* (*Antp*) in the wing imaginal disc. In L3 disc, *Antp* is expressed in the proximal part of the wing disc: the presumptive hinge, pleura and notum (**Figure 13A**; Levine et al., 1983). It is absent from the wing pouch except for a faint signal at the DV border, corresponding to the presumptive region of the adult wing margin where most of the wing sensory organs are specified (see Part 6 of this Chapter for more details). When we look closer at the presumptive notum, a strong staining of Antp is detected in an anterior stripe, which extends from the notum/hinge border to the most dorsal part of the disc, the stalk (**Figure 13B**, arrowhead). Another strong signal, with a circular shape, is observed the most posterior domain of the notum, proximal to the notum/hinge border (**Figure 13B**, arrow). The *Antp* gene has two promoters, *P1* and *P2* (Jorgensen and Garber, 1987). *AntpP1* promoter controls the expression of *Antp* in the anterior stripe of the presumptive notum, evidenced by colocalization between the expression of the *AntpP1-Gal4* driver with the protein (**Figure 13C**, arrowhead); but it is not responsible for the more posterior expression domain (**Figure 13C**, arrow), dependent instead on the *P2* promoter (Jorgensen and Garber,

1987). In a pupa, the expression of the *AntpP1-Gal4* driver is restricted to the anterior part of the thorax, the prescutum (**Figure 13D**).

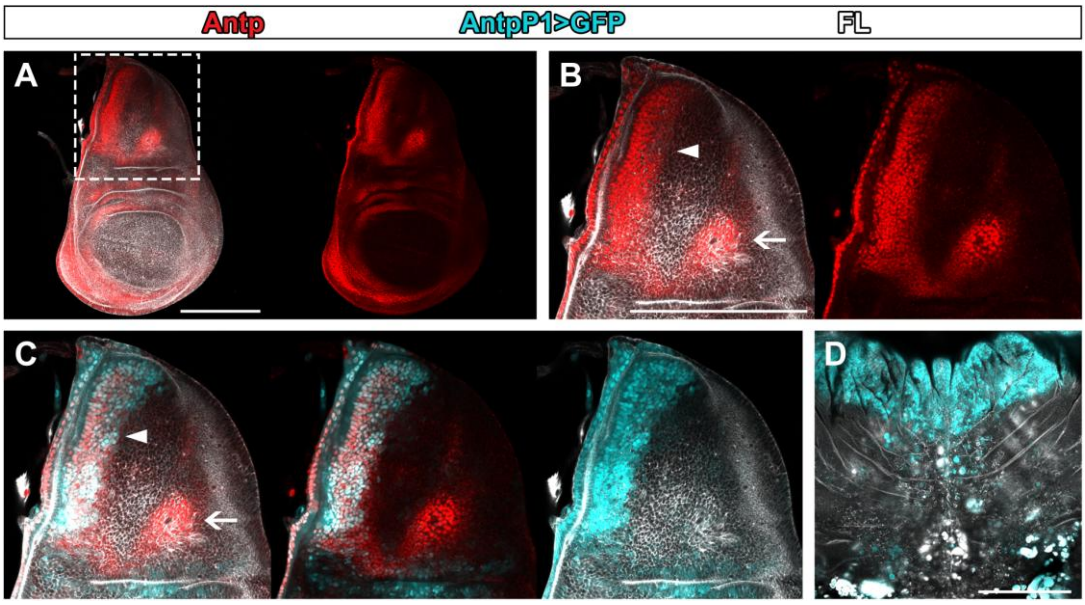


Figure 13. Expression pattern of *Antp* in the presumptive notum. (A) Antp expression is globally restricted to the proximal part of the wing disc. **(B)** A zoom of A panel in the thorax region (white dashed line) showing a strong expression of *Antp* in the most anterior cells of the presumptive notum (arrowhead) and in a more posterior cluster of cells (arrow). **(C)** *AntpP1>GFP* colocalizes with Antp protein in the anterior stripe of cells in the presumptive notum (arrowhead) but it is not expressed in the posterior cluster of Antp-positive cells (arrow). **(D)** Expression of *AntpP1>GFP* in a newly formed thorax revealing an expression in the prescutum. Scale bar: 200 µm. Phalloidin (FL) marks filamentous actin. In (A), (B) and (C) wing discs are oriented anterior-left, dorsal-top; in (D) anterior is towards the top.

2 - Evaluation of the *RNAi-Antp* fly lines

In order to study the role of *Antp* during thorax closure, we have at our disposal two independent and specific *RNAi-Antp* fly lines: a Valium 10 from BDSC, referred as *RNAi-Antp*^{V10} and a KK line from VDRC, referred as *RNAi-Antp*^{KK}. To ensure the effectiveness of these *RNAi*, we tested the reproduction of a known phenotype upon *Antp* removal. *Antp* is expressed in the humeral (or prothoracic) imaginal disc (**Figure 14A**) and its inhibition leads to a lack of eversion of the adult structure (Abbott and Kaufman, 1986). Compared to a wild type fly (**Figure 14B**; the arrowhead point out the humeral), we notice that when we expressed ectopically the *RNAi-Antp*^{V10} in the prothoracic disc, we obtained a partial failure of the eversion of the humeral (**Figure 14C**). However, a complete lack of eversion of the humeral was obtained by expressing ectopically the *RNAi-Antp*^{KK} in this tissue (**Figure 14D**),

indicating that the *RNAi-Antp*^{KK} is stronger than the *RNAi-Antp*^{V10} in knocking down *Antp* function. Also, we ectopically expressed them in random induced clones and observed a change of Antp protein levels. We noticed that the *RNAi-Antp*^{V10} was able to partially decrease *Antp expression* (**Figure 14E**) while the *RNAi-Antp*^{KK} shown better efficiency since no signal was detected (**Figure 14F**), consistent with the adult phenotype previously described. Then, to test the specificity of these lines we expressed ectopically both of them in a tissue where *Antp* is not expressed: we used *eyeless* (*ey)-Gal4* to drive the expression of these *RNAi* in the eye-antenna disc and, as expected, we did not observe a phenotype in the adult head (not shown).

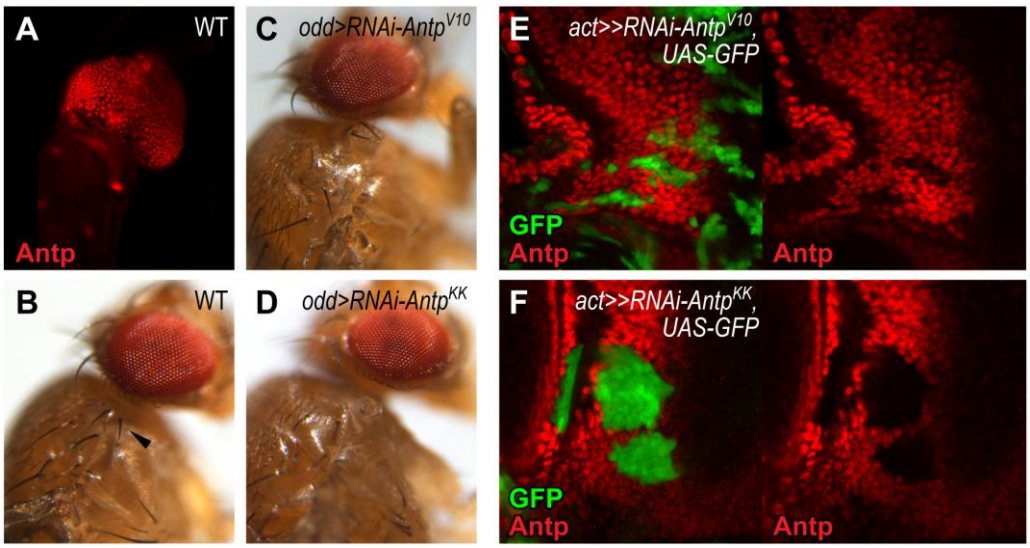


Figure 14. Test of efficiency of the two *RNAi-Antp* lines used. (A) WT expression of *Antp* in the prothoracic disc. **(B)** WT fly. The arrowhead indicates the humeral. **(C)** Ectopic expression, under the control of *odd-Gal4*, of the *RNAi-Antp*^{V10} from BDSC (Valium 10; #27675) leads to a partial eversion of the humeral, whereas **(D)** a complete lack of eversion is obtained with the *RNAi-Antp*^{KK} from VDRC (KK line; #101774). (**E**) Flip-out *RNAi-Antp*^{V10} clones show a partial reduction of *Antp* expression and **(F)** flip-out *RNAi-Antp*^{KK} clones show no *Antp* expression. Pictures in (E) and (F) were taken from the anterior part of the presumptive notum. Flip-out clones are labeled positively by the GFP.

3 - Antp is involved in adult thorax formation

In order to understand the role of *Antp* during notum morphogenesis, we altered *Antp* expression level in the *pnr-Gal4* domain (**Figure 15A** and **B**). When we expressed ectopically *RNAi-Antp*^{V10} in the notum we obtained a mild cleft thorax phenotype; the two-heminota stay far from each at the dorsal midline, especially at the level of the prescutum and the

scutum (**Figure 15C**). The scutellum has a normal shape (although the most posterior scutellum macroachete are often found in a perpendicular orientation to the thorax, probably due to a bad thorax extension during the posteclosion period). These observations indicate a failure in the fusion of the anterior part of the heminotum. Interestingly, the relative pattern of the micro and macrochaete and their number is not affected and no trace of tissue necrosis is apparent (**Figure 15C**). To confirm this result we then expressed ectopically the strong line *RNAi-Antp*^{KK} in the *pnr* expression domain. Consistent with the previous observation for *RNAi* efficiency, a more severe cleft phenotype was observed (**Figure 15D**). Next, to validate the specific requirement of *Antp* during thorax formation, we expressed simultaneously the *UAS-RNAi-Antp*^{KK} and the *UAS-Antp*. Under this condition, we observed a perfect continuous and regular thorax (**Figure 15E**), although the scutellum presents a slight reduction. Note that to avoid diluting the effect of the *Gal4* in this last experiment, the phenotype observed in **Figure 15D** upon *RNAi-Antp*^{KK} ectopic expression has been performed coexpressing a *UAS-GFP*, achieving two *UAS* constructs in both experiments.

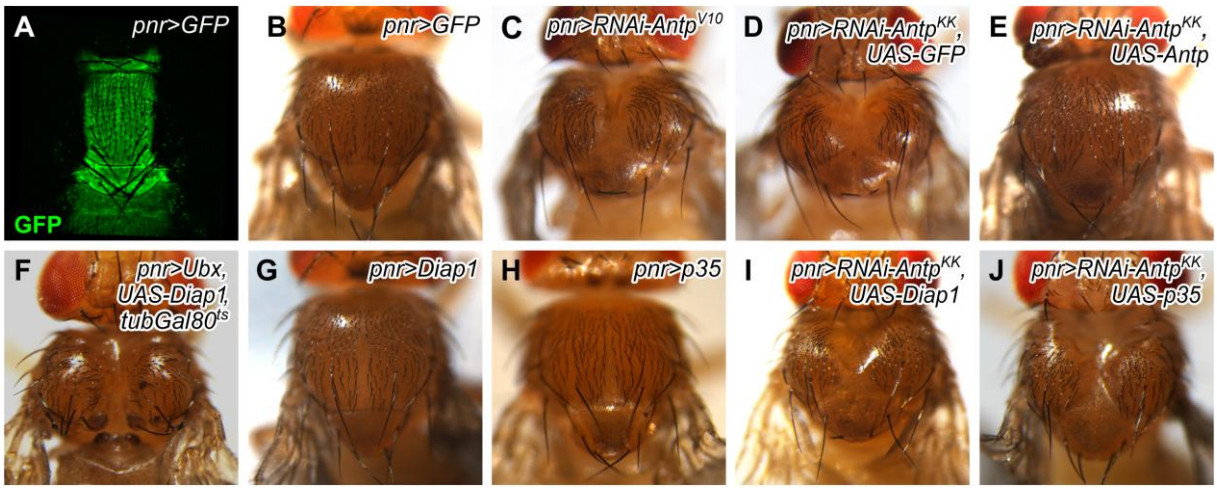


Figure 15. Loss of *Antp* function in the stalk leads to defect in thorax closure in a cell death-independent manner. (A) Expression domain of *pnr>GFP* in a pharate. **(B)** A *pnr>GFP* fly used as control. **(C)** Removing *Antp* function with the *RNAi-Antp*^{V10} (weak *RNAi-Antp* line) in the *pnr* expression domain leads to a modest cleft thorax. **(D)** A more severe phenotype is observed with the *RNAi-Antp*^{KK} (strong *RNAi-Antp* line). Note that one *UAS-GFP* transgene is also present. **(E)** Ectopic expression of *Antp* in the stalk is able to rescue the cleft thorax phenotype caused by the ectopic expression of the *RNAi-Antp*^{KK}, demonstrating the specific implication of Antp in thorax closure. **(F)** Ectopic expression of *Ubx*, a repressor of *Antp*, in the stalk, also leads to a failure in thorax closure. **(G,H)** *pnr>Diap1* (G) and *pnr>p35* (H) do not affect thorax development. **(I,J)** Inhibition of apoptosis with an *UAS-Diap1* (I) or an *UAS-p35* (J) is not able to rescue the phenotype induced by *pnr>RNAi-Antp*^{KK}, showing that prevention of cell death has no effect on the cleft thorax phenotype induced by the lack of *Antp* function. (For all genotype presented here, few variability of the phenotype is observed between individuals, n>50 for each case).

While we were examining the wild type expression pattern of *Antp* in the wing imaginal disc we noticed that *Antp* was also expressed in the Peripodial Epithelium (PE). Its expression colocalizes with Cubitus interruptus (Ci), indicating that *Antp* is expressed in the Anterior (A) cells of the PE (**Figure 16A**). Interestingly, it has been previously described that the expression of Hox gene *Ultrabithorax* (*Ubx*) is restricted to the Posterior (P) compartment of the PE (**Figure 16B**; Pallavi and Shashidhara, 2003, 2005; McClure and Schubiger, 2005). Therefore it appears that these two Hox genes have an exclusive expression pattern in the PE. Given that in the embryo, a posterior Hox represses the expression of the anterior ones (Struhl, 1983; Busturia and Morata, 1988), we tested whether a similar regulation occurs in the PE of the wing imaginal disc between *Antp* and *Ubx*. In one hand, we found that the removal of *Ubx* function in mutant clones leads to a derepression of *Antp* (**Figure 16C**), while the ectopic expression of *Ubx* in the *pnr* expression domain or in random induced clones was able to repress *Antp* expression in the notum or in the A compartment of the PE (**Figure 16D** and not shown). This result indicates that Ubx is necessary and sufficient to repress *Antp* expression. Therefore, we postulated that *Ubx* ectopic expression in the stalk would mimic the adult phenotype induced by the lack of *Antp* function. Since we noticed that ectopic expression of *Ubx* in the stalk induces cell death (not shown) we overcame this effect by

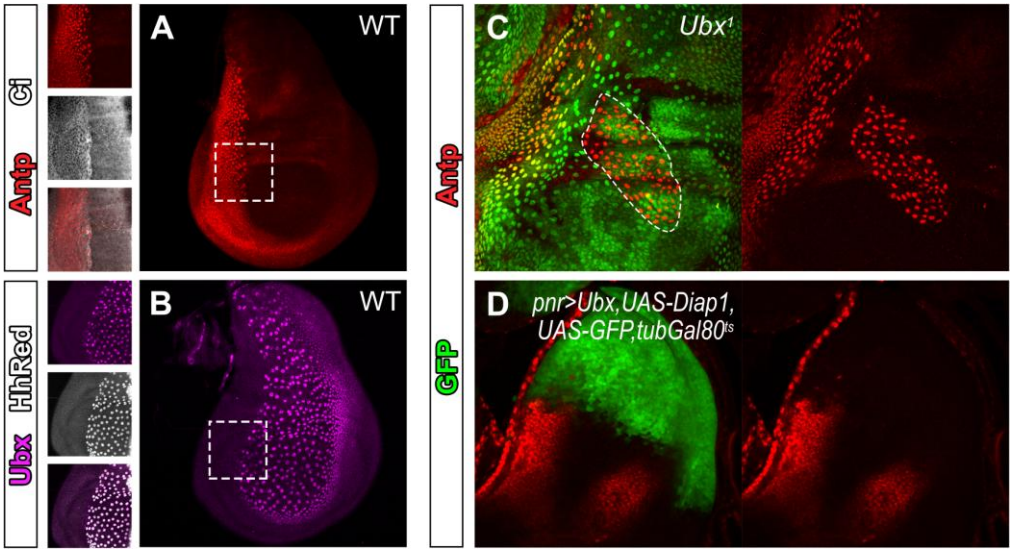


Figure 16. Ubx represses *Antp* expression in the PE. (A) WT expression of *Antp* in the PE. *Antp* expression is restricted to the A compartment as it is shown by the coexpression of Ci (left inserts). **(B)** WT expression of *Ubx* in the PE. *Ubx* expression is restricted to the P compartment since it is coexpressed with a nuclear reporter of Hh expression (HhRed in left inserts). **(C)** An Ubx^{1} mutant clone (labeled by the lack of GFP) showing a derepression of *Antp* in the posterior compartment of the peripodial epithelium (100%, n=18). **(D)** Ectopic expression of *Ubx* in *pnr>Ubx,UAS-Diap1,tubGal80^{ts}* shows a repression of *Antp* in the notum. Time of induction of ectopic expression: 30h. Wing discs are oriented anterior-left, dorsal-top.

expressing it ectopically while inhibiting apoptosis. Under this condition, flies also presented an even more severe cleft thoracic phenotype (**Figure 15F**).

As presented in **Figure 15C** and **D**, the notum of *pnr>RNAi-Antp* adult flies never show signs of necrosis or changes in the notum bristles, although each heminotum keeps apart, the notum presents a correct patterning. These observations suggest that the failure of dorsal closure in the absence of *Antp* is not mediated by apoptosis. In a wild type situation, closure occurs in absence of cell death and inhibition of cell death does not alter adult thorax development (**Figure 15G** and **H**; Martin-Blanco et al., 2000; Joza et al., 2008; Yoshioka et al., 2008). When we expressed ectopically *RNAi-Antp*^{KK} with either *Diap1* or *p35* we were still able to see a cleft thorax phenotype (**Figure 15I** and **J** respectively), similar to the one observed upon ectopic expression of *Antp-RNAi*^{KK}. All together, these data indicate that the failure of heminota fusion due to a reduction of *Antp* levels is not due to apoptosis.

4 - Antp controls actin cytoskeleton remodeling during thorax closure

Thorax closure starts when the two presumptive heminota, initially on a lateral position, crawl over the larvae cell, get in contact by the stalk and intermingle according to a stereotype patron. First, anterior leading edge cells move towards the midline (5.5h APF; **Figure 17A1**), next posterior leading edge cells come close to each other (6.0h APF; **Figure 17A2**), and then middle cells fuse (6.5h APF; **Figure 17A3**) (Martin-Blanco et al., 2000). At this late stage an actin cable is observed at union between both heminota which then fates gradually to disappear in the mature notum (7.0h APF; **Figure 17A4**). Migration and zippering steps are highly dynamic processes and are promoted by a change of actin cytoskeleton distribution and by sending out actin-based filopodia from the stalk (Zeitlinger and Bohmann, 1999; Martin-Blanco et al., 2000; Pastor-Pareja et al., 2004). To know whether actin organization is impaired when *Antp* function is removed we expressed ectopically the *RNAi-Antp*^{KK} in the *pnr-Gal4* domain, whose expression domain covers the entire stalk. As *Antp* expression is restricted to the anterior notum cells, the posterior cells were used as internal control. At 5.5h APF *pnr-Gal4 RNAi-Antp*^{KK} posterior cells of the leading edge showed an evident cell surface enlargement and a strong actin accumulation. Conversely, anterior cells present a poor shape change and no actin accumulation. Anterior parts of the heminotum are away from each other (**Figure 17B1**). At 6.0h APF posterior part

of the heminotum start fusing and leading edge cells emit filopodia. By contrast, the anterior parts remain far from each other and leading edge cells present the same morphology than cells far from the stalk (**Figure 17B2**). At 6.5h APF, middle cells of the stalk get close while emitting filopodia. Anterior heminotum are juxtaposed but no sign of actin recruitment is visible (**Figure 17B4**). At 7.0h APF, posterior thorax has a continuous morphology but the anterior parts present a split shape with smooth borders. Depending on the preparation, a gap is visible between the two anterior heminotum halves (**Figure 17B4** and **B4'**).

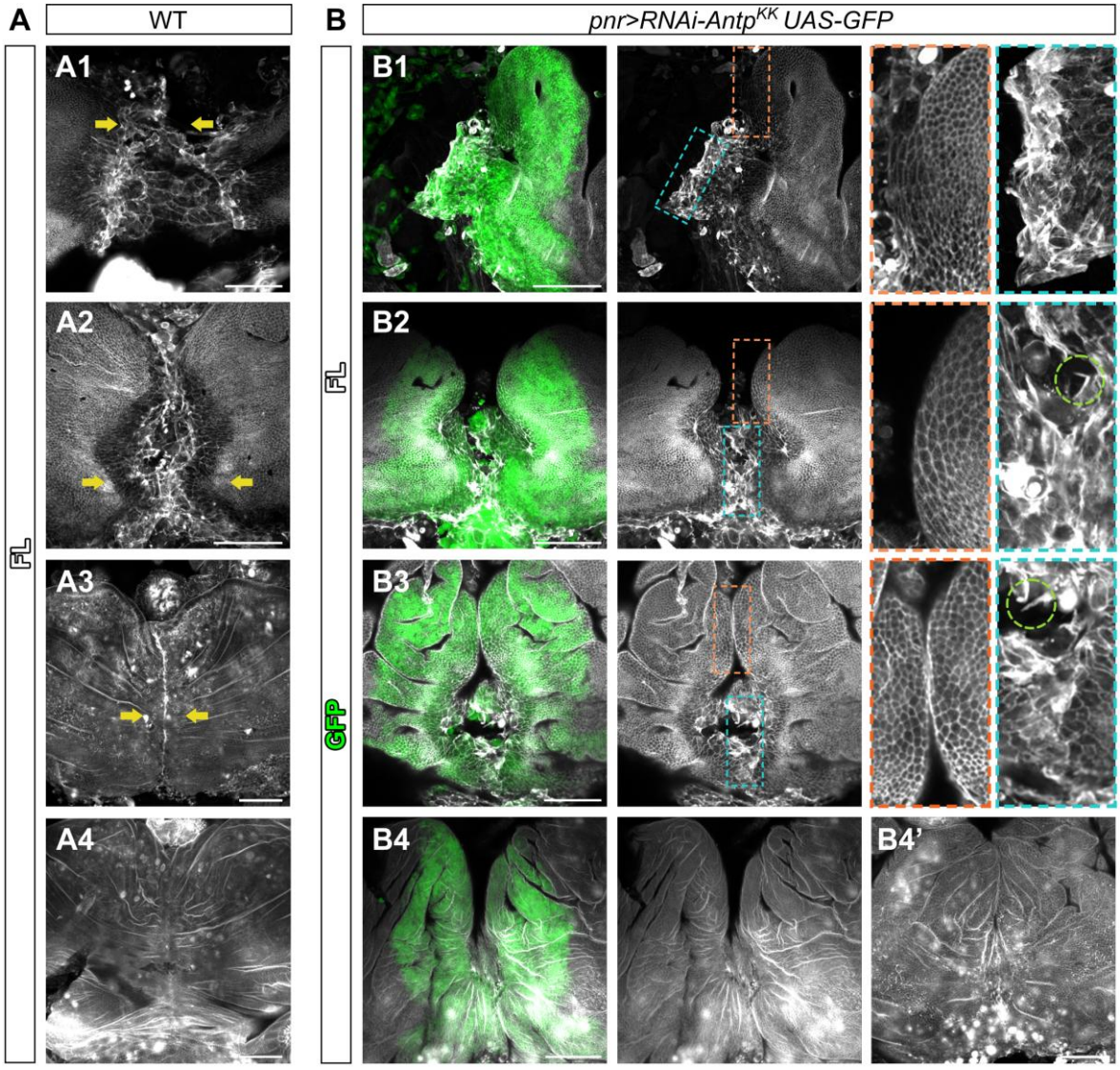


Figure 17. Antp regulates actin organization in the cells of the leading edge. (A) WT thorax closure temporal sequence. The arrows point out the sequential rapprochement of the couple of discs. **(B)** Thorax closure of *pnr-Gal4 RNAi-Antp^{KK} UAS-GFP* pupae. The leading cells of the anterior heminota neither change their shape nor present an accumulation of actin while the ones of the posterior heminota elongate of their area and strongly recruit actin. Only the posterior cells of the leading edge emit filopodia (greenish circles). A1,B1: 5.5h APF; A2,B2: 6.0h APF; A3,B3: 6.5h APF; A4,B4,B4': 7.0h APF. Scale bar: 100 µm.

5 - Antp does not regulate Dpp signaling but it does JNK signaling in the stalk

Dpp is one of the signaling pathways known to control thorax closure (Spencer et al., 1982; Morimura et al., 1996; Simin et al., 1998; Hudson et al., 1998; Chen et al., 1998). Dpp expression at the AP border is downstream of Hh signaling and inherited from the embryo. In early L3 it activates the expression of *pnr* and its antagonist *u-shaped* (*ush*) in the stalk. During L3 stage while the disc grows the *pnr* expression domain extends more ventrally than the *Ush* expression domain. In this *Pnr+/Ush-* domain, Pnr is able to activate its targets *wg* (Tomoyasu et al., 2000; Sato and Saigo, 2000). In addition, it has been reported that Hh signaling, as Dpp signaling, regulates negatively the expression of the Pax-homeobox gene *eyegone* (*eyg*) in the notum (Aldaz et al., 2003).

Wild type expression of *dpp-lacZ*, *wg* and *eyg* (**Figure 18A-C** respectively), was unchanged in *pnr>RNAi-Antp*^{KK} disc (**Figure 18D-F** respectively). The dorsocentral (DC) enhancer drives the *achete*/*scute* complex (AS-C) expression in the proneural clusters that give rise to the four DC macrochaeta on the scutum. This enhancer is known to be controlled by Dpp, Pnr, Ush and Wg (reviewed in Gomez-Skarmeta et al., 2003). Although we have not directly tested the expression of the *AS-C* in the wing imaginal disc we observed that, as previously mentioned, *pnr>RNAi-Antp* flies do not present an alteration of the bristle patterning (**Figure 15C** and **D**). All together these results indicate that Antp does not act upstream of Dpp signaling.

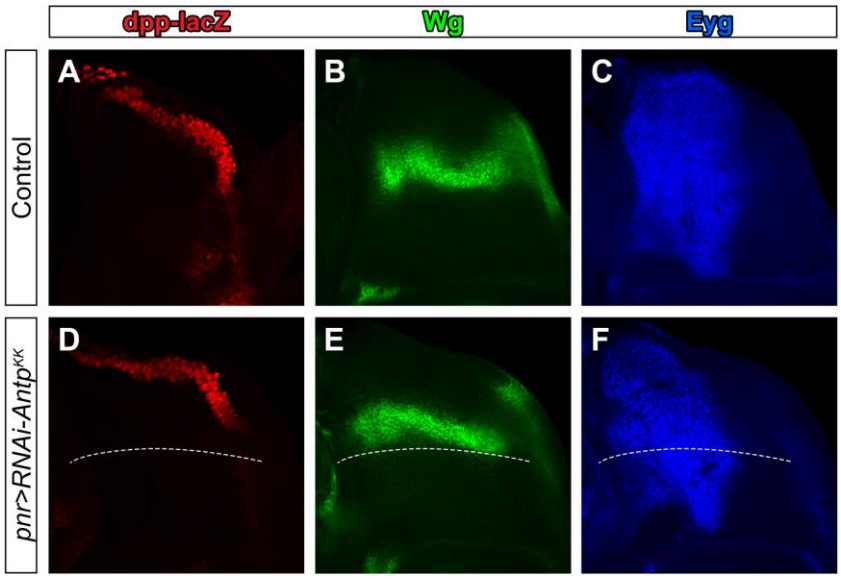


Figure 18. Antp does not regulate Dpp signaling in the notum. WT expression of *dpp-lacZ* **(A)**, Wg **(B)** and Eyg **(C)** in the presumptive notum. Removing *Antp* function in *pnr>RNAi-Antp*^{KK} flies does not affect *dpp-lacZ* **(D)**, Wg **(E)** nor Eyg **(F)** expression. The dashed line in (D-F) delimits the *pnr-Gal4* domain.

c-Jun N-terminal kinase (JNK) signaling is a well-known signaling pathway implicated in thorax fusion (Zeitlinger and Bohmann, 1999; Martin-Blanco et al., 2000). In a wild type situation, *puckered* (*puc*)-*lacZ*, a reporter of the activity of this signaling pathway is expressed in the stalk. We monitored JNK activity by examining *puc-lacZ* expression upon alteration of *Antp* expression. A change in *puc-lacZ* expression is observed when decreasing *Antp* expression in random induced clones. Interestingly, only the most dorsal cells of the mutant clones present an ectopic expression of *puc-lacZ*, besides this effect appears only at the edge of the clone both in an autonomous and a non-autonomous manner (**Figure 19A**). To evaluate the implication of the JNK pathway in the cleft thorax phenotype mediated by *Antp* downregulation, we took advantage of the fact that Puc acts as a repressor of the pathway by dephosphorylating the upstream kinase Basket (Bsk). We found out that ectopic expression of *puc* in *Antp*-downregulated cells does not activate *puc-lacZ* expression in clones located close to the stalk (**Figure 19B**). Similar results were obtained when we blocked Bsk function in *Antp*-downregulated cells (**Figure 19C**). All together these results reveal that the cleft phenotype produce by *Antp* downregulation alters the JNK pathway but not Dpp signaling pathway.

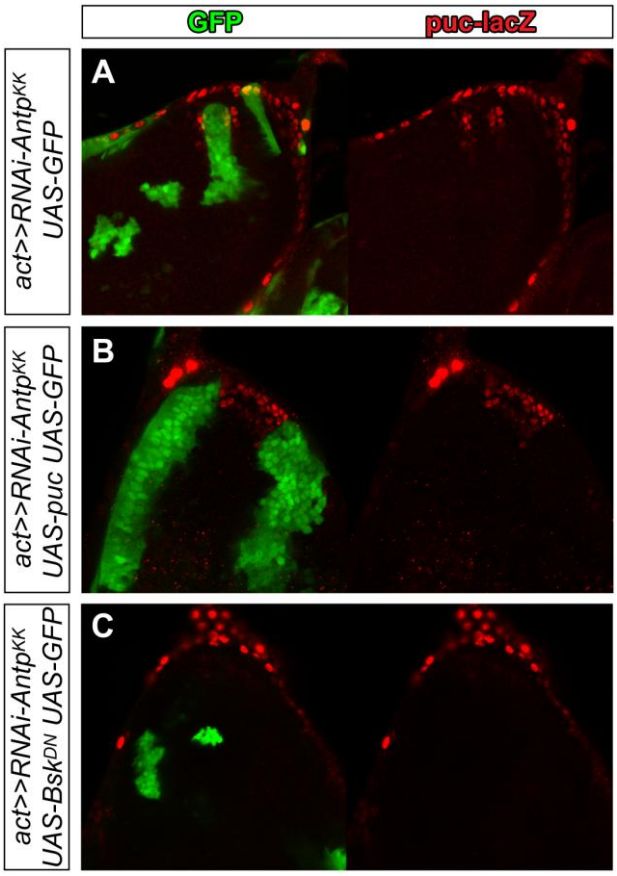


Figure 19. Antp regulates JNK signaling in the stalk. Downregulation of *Antp* expression in the notum leads to an ectopic activation of *puc-lacZ* in the most dorsal cells of the stalk in an autonomous and a non-autonomous manner **(A)**. This effect is rescued by expressing ectopically *puc* itself **(B)** or a Bsk^{DN} construct **(C)**. Flip-out clones are marked positively by the GFP. ((A) 80% n=10, (B) 100% n=11, (C) 100% n=10).

6 - *Antp* missexpression affects wing sensory organs formation

We finish the first Chapter of this Thesis by presenting results related to an *Antp* function in the formation of the wing sensory organs, different from its role during dorsal closure. It seemed appropriated to us to report these preliminary findings in this Thesis, especially given the fact that to date no study has linked the Hox gene *Antp* to sensory organs formation. While we were analyzing the role of *Antp* in thorax closure, we noticed that, in addition to the expression of *Antp* in the notum (**Figure 13**), the *AntpP1* promoter shows an expression in the presumptive wing margin (**Figure 20A**) and the Antp protein is also detected in these cells (**Figure 20B**). To make sure of the specificity of this signal, we expressed ectopically the *Antp-RNAi*^{KK} in the wing pouch. We observed that, in this condition, *Antp* expression was lost at the DV border, indicating that *Antp* is indeed expressed at the presumptive wing margin (**Figure 20C**). *AntpP1* expression has already been partially described (Jorgensen and Garber, 1987), as the authors only reported an expression of the *AntpP1* promoter in the anterior compartment of the presumptive wing margin.

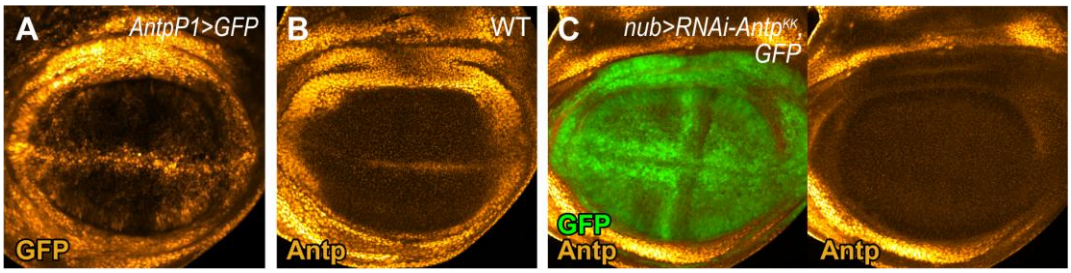


Figure 20. *Antp* is expressed all along the wing margin. Wing margin cells express the *AntpP1* driver **(A)** and the Antp protein **(B)**. This later expression is lost when the *RNAi-Antp*^{KK} is ectopically expressed in the wing pouch **(C)**. Wing discs are oriented anterior-left, dorsal-top.

The adult wing margin carries several kinds of sensory organs: the recurved bristles corresponding to chemoreceptor organs and the stout and slender bristles, two mechanoreceptor organs. Another sort of mechanoreceptors with a dome-like shape structure, the campaniform sensilla, is present on the wing blade at the level of the third vein (**Figure 21A**; red arrows). When we expressed ectopically *Antp* in the entire wing pouch we notice an evident alteration of sensory organ pattern. Indeed, we observed campaniform sensilla not only in the L3 vein but also associated with other veins (**Figure 21B**; orange arrows). Ectopic slender bristles were also seen all over the wing blade (**Figure 21B**; purple arrowheads). This data indicate that ectopic *Antp* expression in the wing pouch is able to

specify sensory organs. However, we do not know whether these organs are functional, and especially whether the slender bristles are innervated. Finally, we noticed that the wing margin carry less sensory organs, which affects to recurved, stout and slender bristles (**Figure 21B**; see green area). It remains to be determined whether the abnormal pattern of bristles at the wing margin is due to a lack of proneural cluster formation or rather an alteration in the sensory organ specification. Given that in a wild type situation *Antp* is expressed in the wing margin cells, an area rich in chemo and mechanoreceptors, and that ectopic *Antp* expression is sufficient to alter wing sensory organ patterning, it will be interesting to investigate at which level the endogenous levels of Antp are involved in sensory organ development.

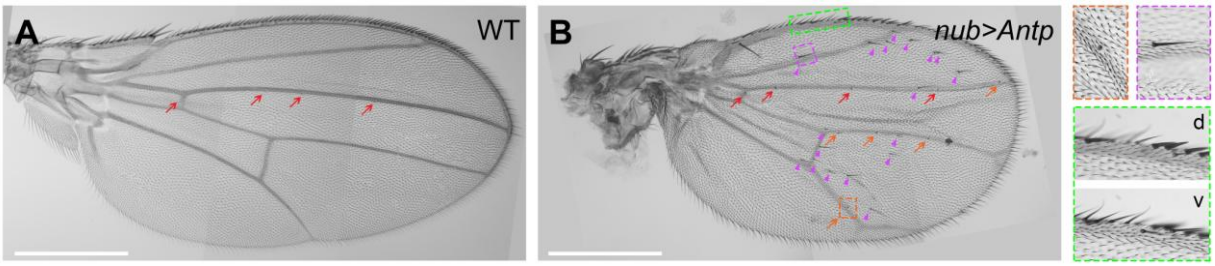


Figure 21. Ectopic expression of *Antp* in the wing alters sensory organ formation. (A) WT wing. Red arrows point out the four campaniform sensilla present on the L3 vein. **(B)** *nub-Gal4 UAS-Antp* wing shows ectopic campaniform sensilla on the wing veins (orange arrows). Endogenous campaniform sensilla are marked by red arrows. Ectopic stout bristles (pink arrowheads) are specified all over the wing blade. Pattern of margin bristles (stout, slender and recurved) is aberrant (green rectangle). Right inserts are zooms of an ectopic campaniform sensillum, an ectopic stout bristle and of the dorsal (d) and ventral (v) margin. Note also that the wing area is reduced compared to WT. Scale bar: 500 µm.

Discussion

1 - Antp and thorax closure

Adult thorax derives from the fusion of two heminota. During the metamorphosis, the pair of imaginal wing discs, initially present on the larva flanks, starts elongating and the most dorsal parts move toward the dorsal midline. Cells of the stalk fuse and intermingle to form a continuous epithelium in a zippering manner without involving cell death (Martin-Blanco et al., 2000; Joza et al., 2008; Yoshioka et al., 2008). The cells that initiate the migration step are the most anterior cells of the presumptive notum, corresponding to the prescutellum. Precisely, *Antp* is expressed in a stripe of the anterior presumptive notum (**Figure 13**). This expression pattern led us to wonder whether this *Antp* expression could be involved in thorax closure. To this aim, we knocked down *Antp* expression by expressing specific *RNAis-Antp* in the dorsal region of the wing disc. An obvious cleft phenotype could be observed, and interestingly the lack of dorsal closure was restricted to the anterior notum; the posterior part of the notum was correctly formed (**Figure 15C** and **D**). The specificity of the *RNAi-Antp* effect could be confirmed by rescuing the phenotype after overexpressing the protein (**Figure 15E**). Accordingly, a cleft thorax phenotype is observed by expressing

ectopically *Ubx* (**Figure 15F**), a repressor of *Antp* expression in the wing disc (see part 3 of the Discussion; **Figure 16C** and **D**).

We found that the lack of thorax closure upon a decrease in *Antp* function is not a consequence of cell death induction (**Figure 15I** and **J**). We attribute this phenotype to a missexpression of JNK signaling (**Figure 19**), a signaling pathway known to be involved in this morphogenetic process by controlling cytoskeleton rearrangement (Martin-Blanco et al., 2000). Besides, we could see that when *Antp* function is downregulated in the stalk, leading edge cells do not accumulate actin and do not emit filopodia towards the middle line (**Figure 17**). It is not the first time that Hox gene function is associated to cytoskeleton organization. Indeed, it has been shown that they can be in charge of the compartment boundary maintenance by regulating actomyosin cortex (Curt et al., 2013). Interestingly, the homolog of *Antp mab-5* in the nematode *Caenorhabditis elegans* is responsible for neuroblasts descendants' migration (Salser and Kenyon, 1992; Harris et al., 1996), a process that requires actin remodeling.

A morphogenetic process similar to adult thorax closure occurs in the embryo, referred as dorsal closure. After retraction of the germ band, the lateral epidermis from both sides of the embryo migrates at the midline concomitantly to the delamination of the cells of the amnioserosa. Leading edge cells, localized at the interface between the epidermis and the amnioserosa, present functional similitude to the wing disc stalk by exerting a motor force over an entire tissue (Kiehart et al., 2000). However, removing *Antp* function or expressing ectopically *Ubx* in the embryo does not alter embryo dorsal closure, only alters segmental identity (Wakimoto and Kaufman 1981; Gonzalez-Reyes and Morata, 1990), indicating that Antp is only involved in epithelial sheet movement during post-embryonic stage.

2 - Hox genes: beyond homeotic transformation

From a developmental biology point of view this new role assigned to a Hox gene appears to be somehow atypical considering the traditional association of Hox selector function (Garcia-Bellido, 1975). Indeed, gain- or loss-of-function of Hox gene are known for reprogramming the fate of an organ. Two obvious illustrations where Hox genes act as master control genes for identity (Gehring, 1996), among many other reported homeotic transformations, are the

transformation haltere-to-wing by removing *Ubx* function in the haltere (Morata and Garcia-Bellido, 1976; Lewis, 1978), or again the homeotic transformation antenna-to-leg by expressing ectopically *Antp* in the antenna (Postlethwait and Schneiderman, 1971). However, Hox gene function is not only restricted to an identity input. For example, removing the expression of Hox *Dfd* in the maxillary palp primordia results in the deletion of the adult appendage (Merrill et al., 1987; Pultz et al., 1988). Likewise, removing *Antp* function in the prothoracic disc leads to defects in the eversion of the humeral (Abbott and Kaufman, 1986). Our data show that *Antp* removal from the presumptive notum does not result in a transformation of notum cells into a different fate but rather in a tissue dynamic alteration during the morphogenesis of the adult thorax. This result constitutes therefore one of the few example in which Hox function is not related to a homeotic identity.

It would be interesting to investigate whether this novel function of *Antp* is conserved in other insects. Actually, it has been attempted to remove *Antp* function from the larvae butterfly *Bicyclus anynana* (by injecting RNAi), however no effect on adult morphology was visible, probably due to low efficiency of RNAi technique in Lepidoptera (Saenko, 2010). We have to keep in mind, however, that although Hox genes are expressed in a similar fashion along the AP axis between Diptera and Lepidoptera, their function can diverge between homologous segments. Indeed in both orders, *Ubx* is expressed in the T3 segment but in the fly it imposes the haltere *versus* the wing fate whereas in the butterfly, a hindwing arises from the T3 segment (Saenko et al., 2011).

3 - Hox genes expression in the PE is a readout of the wing imaginal disc primordium position in the embryo

Wing primordium is specified during embryogenesis at the boundary between the parasegments (PS)4 and PS5, which corresponds to the limit of expression between *Antp* and *Ubx*. According to the posterior suppression mechanism, *Ubx* mutant embryos lead to a posterior extension of the *Antp* expression domain (Wakimoto and Kaufman, 1981). We uncover that similar regulation occurs in the Peripodial Epithelium (PE). Indeed, we found out that *Antp* and *Ubx* are expressed in the anterior and posterior compartments of the PE respectively (**Figure 16A** and **B**; Pallavi and Shashidhara, 2003) and that this complementary expression pattern is due to a repression of *Antp* by Ubx. Indeed, mutant clones for *Ubx* in

the posterior compartment of the PE lead to a derepression of *Antp* (**Figure 16C**). This regulation does not seem to be relevant regarding wing development but it reflects its embryonic origin. In the Disc Proper (DP), however, *Ubx* repression by Engrailed (En) and by the Polycomb genes (Pc) is a prerequisite to select the wing *versus* haltere fate (Emerald and Shashidhara, 2000; Ingham, 1985). In addition, we have tested whether Antp represses the expression of the preceding Hox gene *Scr*. However we did not see an ectopic expression of *Scr* in Antp mutant cells either in the PE or in the DP (not shown).

4 - Clues for Notch signaling regulation by Antp

We also found out that *Antp* is translated all along the dorsoventral margin of the wing disc and that this expression depends on the *P1* promoter (**Figure 20A** and **B**). This expression had been partially described and the authors reported the *P1* promoter expression in the anterior part of the presumptive wing margin (Jorgensen and Garber, 1987). The dorso-ventral boundary contains Sensory Organ Precursors (SOP), which give rise to the sensory bristles of the wing margin. Our gain of function experiment (**Figure 21**) suggests that Antp could be implicated during the lateral inhibition process, which leads to the selection of the SOP. During this process, the SOP get specified by turning off Notch (N) signaling, and by contrast N signaling is turned ON in its neighboring cell of the proneural cluster. To my knowledge, *Drosophila*, as a model system, has never been used to study the regulation of N signaling by Antp. However, there are more clues favoring this hypothesis. Indeed, it has been reported that induction of mutant clones of *Antp* in wing imaginal disc leads to some missing bristles in the mesonotum adjacent to the humeral callus (Abbott and Kaufman, 1986); some bristles are also missing in similar clones induced in the T1 and T3 legs (Struhl, 1982). In the butterfly, both *Antp* and *N* are expressed in the eyespots wing but no functional regulation has been established yet (Saenko et al., 2011). In human, N signaling is activated in a specific zone of the prostate corresponding to the intermediate cells undergoing proliferation, and missregulation of this pathway can lead to prostate cancer (Wang et al., 2006; Ceder et al., 2008), an organ where the human homolog of *Antp*, *Hox6c*, is expressed (Ramachandran et al., 2005). In addition, it is known that Hox6c is a regulator of N signaling (McCabe et al., 2008). Therefore, it may be worth considering *Drosophila* bristles specification by Antp as a simple model to understand an eventual regulation of N signaling by Antp.

Chapter II

The zinc finger protein Odd paired is implicated in *Drosophila* adult postecdysis by regulating *Bursicon* expression

Results

1 - Expression of *opa* through CNS development

The CCAP neurons are central regulators of the ecdysis process (reviewed in White and Ewer, 2004). They have an embryonic origin and represent a population of some tens of cells mainly found in the VNC; each hemineuromere has one or two CCAP. Most of them get hormonally differentiated (the hormonal differentiation corresponds to the onset of the neuropeptide CCAP expression) at the end of embryogenesis and the few remaining, located at the posterior part of the VNC, differentiate hormonally at the entrance to pupa (Veverytsa and Allan, 2012). When we coexpressed *CCAP>GFP* in *opa-lacZ* flies (see **Figure 27A** for *lacZ* insertion) we observed that all the CCAP cells express *opa-lacZ* in larval (**Figure 22A** and **B**), pupal (**Figure 22C** and **D**) and adult stages (**Figure 22E**). Interestingly, we also noticed that *opa* is already expressed at L2 in the posterior CCAP neurons which start expressing *CCAP-Gal4* at a later stage (see brackets in **Figure 22A, B** and **C**). Similarly, in the embryo, we could already detect *opa* expression at stage (st) 12, while expression of the *CCAP-Gal4* starts later and progressively in the embryonic abdominal segments and earlier in the thoracic segments (**Figure 23**), indicating that *opa* is probably expressed as soon as the CCAP neurons are born.

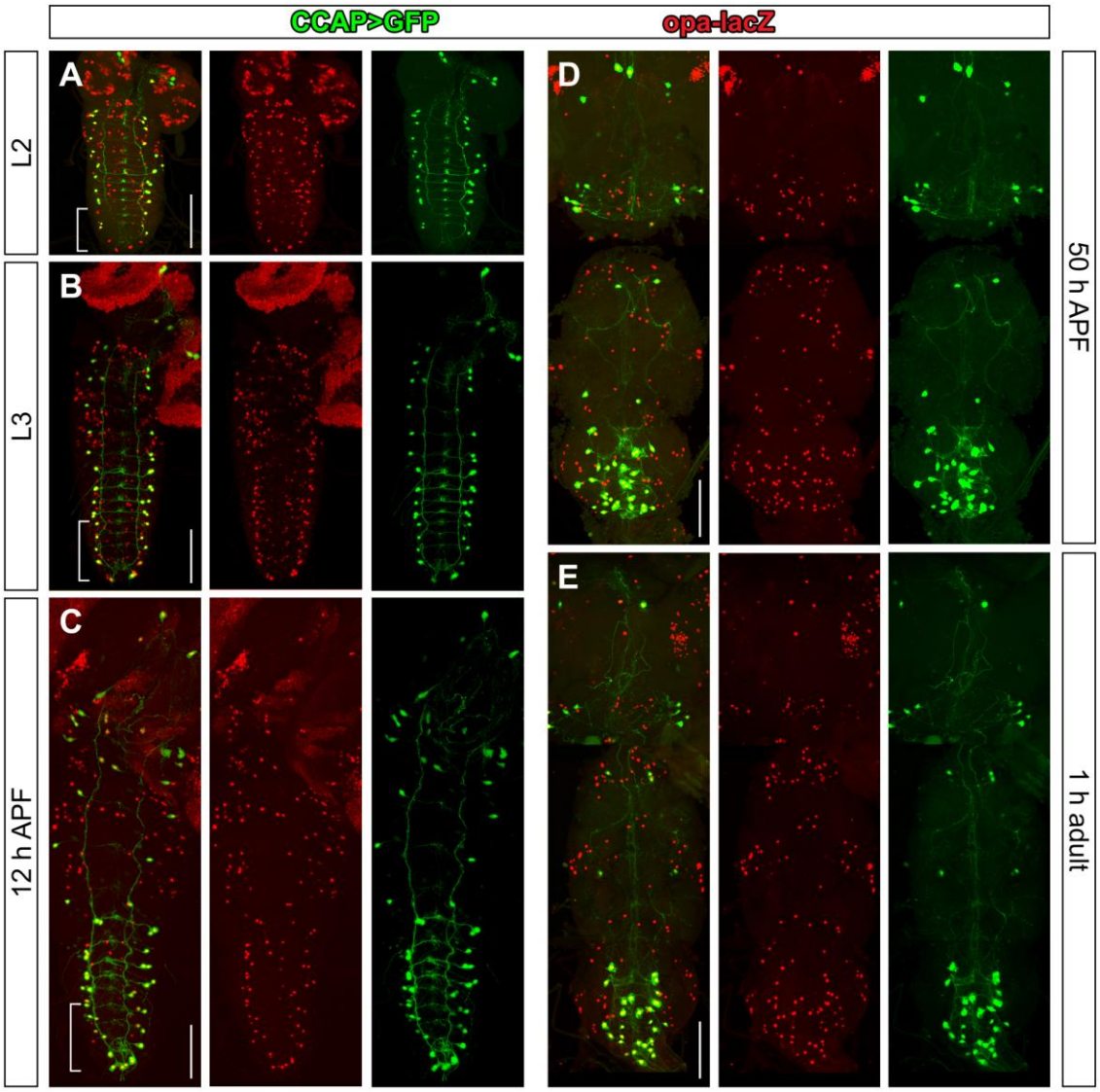


Figure 22. Opa is expressed in the CCAP neurons through development. (A) L2, **(B)** L3, **(C)** 12 h APF, **(D)** 50 h APF and **(E)** 1 h adult CNS showing that all the CCAP neurons express *opa-lacZ*. Pictures correspond to maximum projections of stacks of the entire CNS along the dorsoventral axis. Note that the expression of *opa* (A; bracket) is previous to the expression of *CCAP-Gal4 UAS-GFP* in the most posterior CCAP cells (B and C; bracket). Scale bar: 100 µm. Anterior is towards the top.

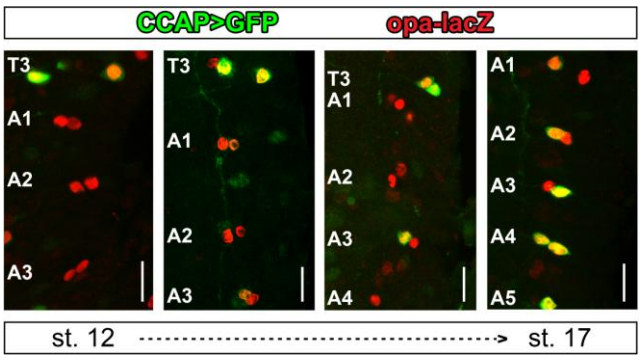


Figure 23. Comparison of *opa* and *CCAP* expression in the CCAP neurons during the embryogenesis. *opa* is expressed at least since st 12 in the CCAP neurons prior to their hormonal differentiation. *CCAP* expression is not synchronized but rather progressive throughout the CCAP neurons population. Scale bar: 10 µm. Anterior is towards the top.

Overall, these results indicate that *opa* expression is maintained through development in the CCAP cells and constitutes a good marker of the CCAP cells independently of the state of the hormonal differentiation.

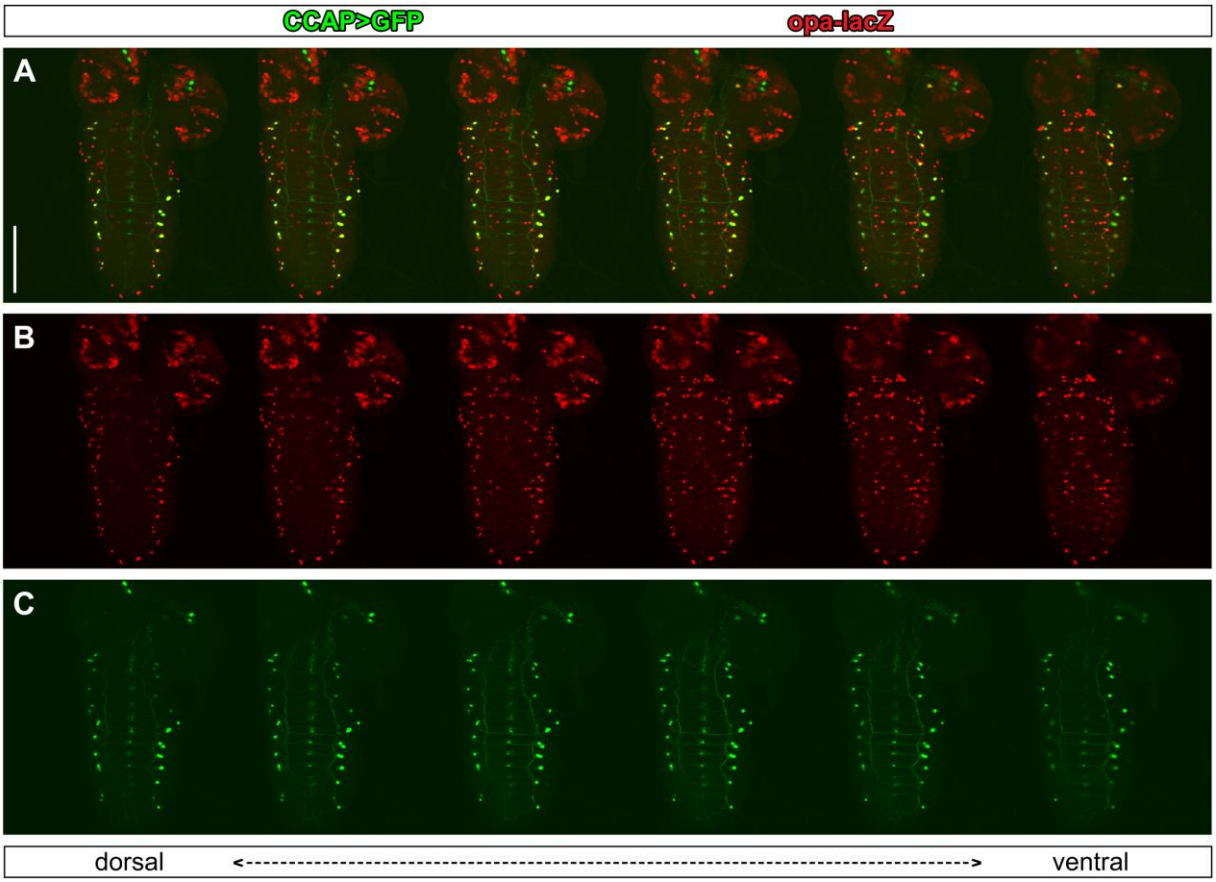


Figure 24. Expression of *opa* along the dorsoventral axis of a L2 CNS. In the dorsal VNC, *opa* is expressed in a segmented manner coinciding to its expression in the CCAP neurons in the thoracic and abdominal neuromeres. In the ventral VNC, *opa* is expressed in the CCAP neurons of the suboesophagic domain. It is also expressed in more ventral neurons of the suboesophagic region and in some ventral abdominal neurons. Images correspond to the frames of the maximal projection presented in Figure 22A. Scale bar: 100 µm. Anterior is towards the top.

Confocal sections along the dorsoventral axis of the CNS (**Figure 24**) show that in a dorsal plane *opa* is exclusively expressed in the CCAP neurons of the thoracic and abdominal segments, while in the ventral side, *opa* is expressed in the CCAP neurons of the suboesophagic region but also in other cells. We attempted identification of these other *opa*-expressing neurons, however, we did not found a particular correlation with known neurons subtypes. Nevertheless, we found that some located in the ventral face of the VNC coexpress the *386-Gal4* line, a peptidergic cell-specific driver (Taghert et al., 2001), at the

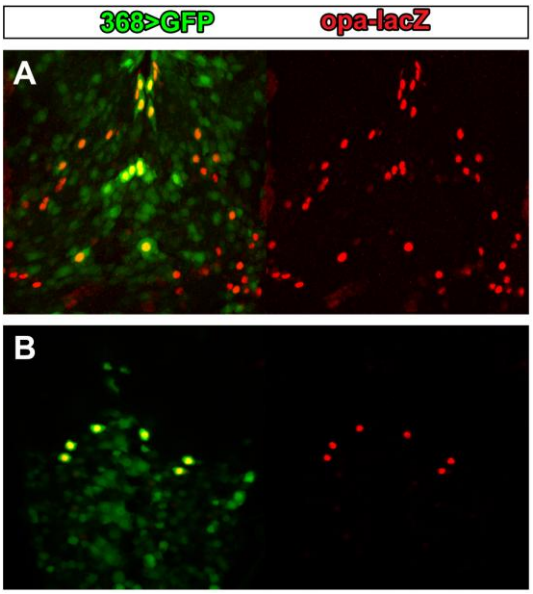


Figure 25. *opa* is expressed in peptidergic neurons different from the CCAP type. *opa* colocalize with the *368-Gal4* driver, expressed in the neurons expressing the neuropeptide myomodulin (O'Brien and Taghert, 1998) in the ventral suboesophagic region **(A)** and in the ventral abdominal segments **(B)**. Pictures are taken from a L3 CNS. Anterior is towards the top.

level of the suboesophageal region (**Figure 25A**) and the abdominal segments (**Figure 25B**), indicating that *opa* is expressed in more than one type of peptidergic cell.

In addition, we also observed that *opa* is strongly expressed through development in some lineages of the Central Brain (CB), in the medulla and in the Optic Lobe (OL) (**Figure 26**), as it has been previously reported (Tix et al., 1997).

The Janelia Farm reported a huge amount of lines expressing *Gal4* under the control of defined putative regulatory sequences. In the case of *opa*, several *Gal4* have been inserted nearby or inside the gene (**Figure 27A**) and their expression profiles are reported on the Janelia farm webpage. The expression profile of *77B01-Gal4* line (coordinate of the inserted fragment: 3R 666795-670479) reproduces quite well the expression of *opa-lacZ* in the CCAP neurons. Interestingly, coexpression of this line in an *opa-lacZ* background reveals that

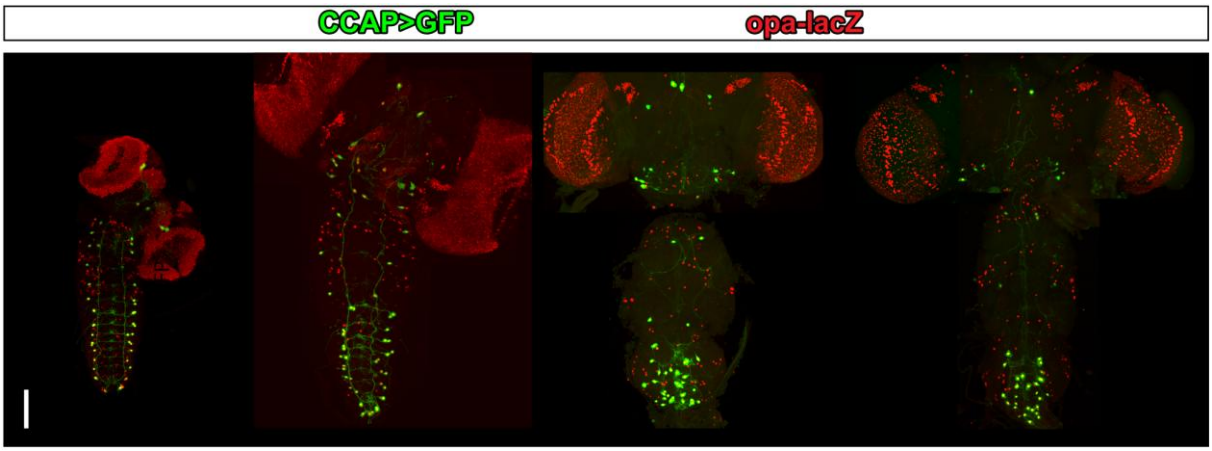


Figure 26. Opa expression in the entire CNS. Pictures correspond to the same CNS maximum projections presented in Figure 22 (from left to right: L3, 12 h APF, 50 h APF and 1 h adult CNS), here including the OL and the entire CB to appreciate the expression of *opa* in the medulla and in various lineages of the CB. Scale bar: 100 µm. Anterior is towards the top.

77B01-Gal4 is expressed principally in the CCAP cells (**Figure 27B**). An upstream and overlapping sequence (3R 665230-667349) expressed in the *77B12-Gal4* line shows poor expression through the CNS (**Figure 27C**). A downstream and overlapping sequence (3R 669104-672762) expressed in the *77B05-Gal4* line recapitulates *opa-lacZ* expression in the OL, the CB and in the most posterior CCAP of the VNC (**Figure 27D**).

Based on these results, it is difficult to determine the exact regulatory sequences required for *opa* expression in the CNS. However, as a preliminary assumption we propose that the expression of *opa* in the CCAP cells (except from the most posterior ones) may be regulated by a sequence of 1,75kb present on the *77B01* fragment and excluded from the *77B12* and *77B05*, corresponding to the sequence: 667350-669103. The expression of *opa* in the most posterior CCAP may depend on a sequence overlapping the *77B01* and the *77B05* fragments

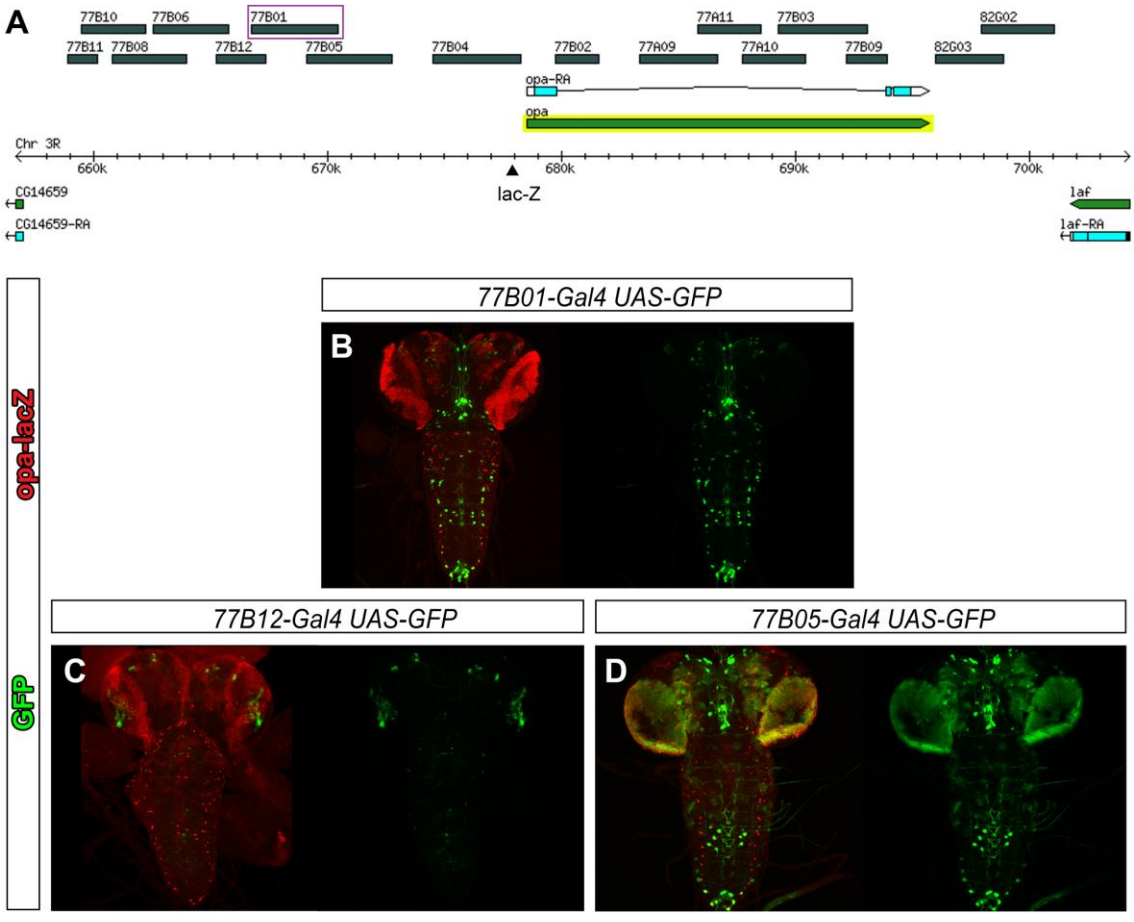


Figure 27. Identification of the DNA fragment that control *opa* expression in the CNS. *opa* gene maps within different fragments cloned by the Janelia Farm **(A)** (adapted from the Janelia FlyLight webpage) showing the expression of the *77B01-Gal4* **(B)**, *77B12-Gal4* **(C)** and *77B05-Gal4* **(D)** in comparison with *opa-lacZ* expression pattern. (See the information related to the *77B01-Gal4* on http://flweb.janelia.org/cgi-bin/view_flew_imagery.cgi?line=R77B01).

(669104-670479). The expression of *opa* in the OL and the lineages of the CB may depend on a sequence included in the *77B05* and excluded from the *77B01*, corresponding to the fragment: 670480-672762.

2 - Evaluation of the *RNAi-opa* lines

To interfere with *opa*, we have at our disposal 3 different *RNAi lines*: one on the X chromosome from VDRC, one on the II chromosome also from VDRC and another one on the III chromosome from BDSC, referred in this thesis as *RNAi-opa*^{X}, *RNAi-opa*^{II} and *RNAi*-opa^{III} respectively. As a preliminary experiment we tested the efficiency of these *RNAi* lines by crossing them with the ubiquitous driver *act-Gal4*. Given that homozygous mutants for *opa* are embryonic lethal, we considered that an efficient *RNAi* would also be embryonic lethal when ectopically expressed in the entire animal. The cross of *act-Gal4* with the *RNAi-opa*^{III} did not result in either lethality or a particular phenotype, not even when using a *UAS-Dicer* (*Dcr*)*-2* to potentiate the RNA degradation machinery, so this *RNAi* was discarded forthwith. The cross of *act-Gal4* either with *RNAi-opa*^{X} or *RNAi-opa*^{II} did not induce lethality at the embryonic stage but rather at late L3 and early L3 stage respectively. This result indicates that these two *RNAi* lines have an effect on development although the lack of embryonic lethality indicates that they do not eliminate completely *opa* function. However, it appears that the *RNAi-opa*^{II} is stronger than the *RNAi-opa*^{X}, thus we mainly used the *RNAi-opa*^{II} for this Thesis.

Furthermore, to ensure the efficiency of the *RNAi-opa*^{II} we tested the expression of *odd-skipped* (*odd*) which we found to be an Opa target in the eye-antenna imaginal disc. In this tissue, *opa* expression is restricted to the proximal part of the antenna, to the presumptive head capsule and to the A1 segment (**Figure 28A**). *odd* is expressed in two concentric rings corresponding to the presumptive joints of the antenna; the most proximal ring is included in the *opa* expression domain (**Figure 28B**). When we induced mutant clones for *opa*^{7}, a null allele of *opa*, the expression of *odd* disappeared (**Figure 28C**, arrow). As we did not get a similar result by the expression of the *RNAi-opa*^{II} alone (not shown) we simultaneously coexpressed it the UAS-*Dcr-2*. As expected, we did see an alteration of *odd* expression in this case (**Figure 28D**, arrows). These results indicate that *RNAi-opa*^{II} is able to downregulate *odd* expression, a newly identified target of Opa, however, it requires the activity of Dcr-2 for an optimal efficiency.

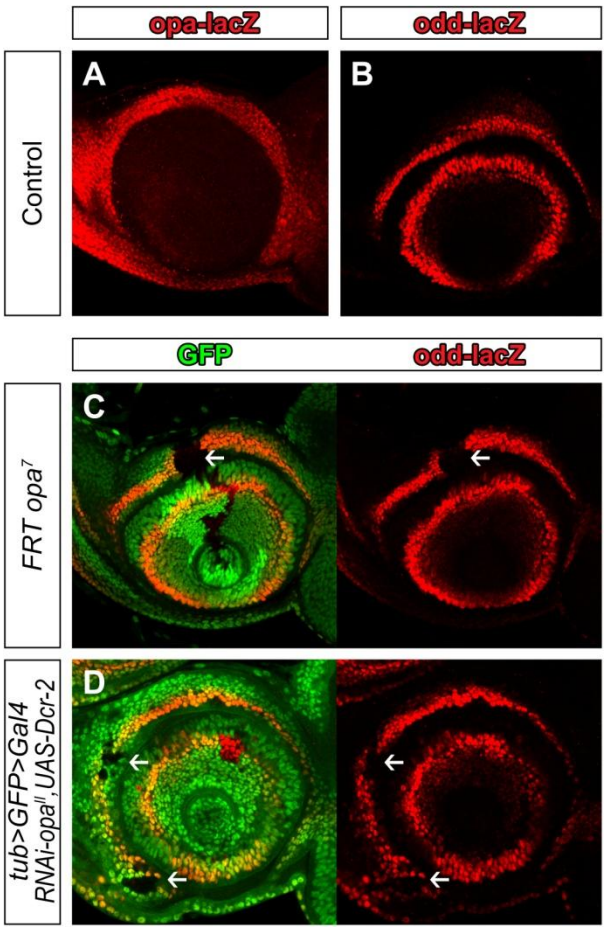


Figure 28. Evidence of *RNAi-opa^{II}* efficiency: *odd* expression is altered upon induction of *opa^{7}* mutant clones and *RNAi-opa^{II}*-expressing clones (A) *opa-lacZ* expression pattern is restricted to the proximal part of the antenna disc. **(B)** *odd-lacZ* expression pattern describes two concentric rings in the antenna disc. The proximal ring of *odd-lacZ* expression is included in the *opa* expression domain. **(C)** Mutant clones for *opa^{7}*, a null allele of *opa*, lead to a loss of *odd-lacZ* expression (arrow). **(D)** Flip-out *RNAi-opa^{II}* clones also lead to a loss of *odd-lacZ* expression (arrows). Note that that in both (C) and (D) *odd-lacZ* expression is only absent in its proximal ring, where *odd* and *opa* coexpress. The clones are marked by the lack of GFP.

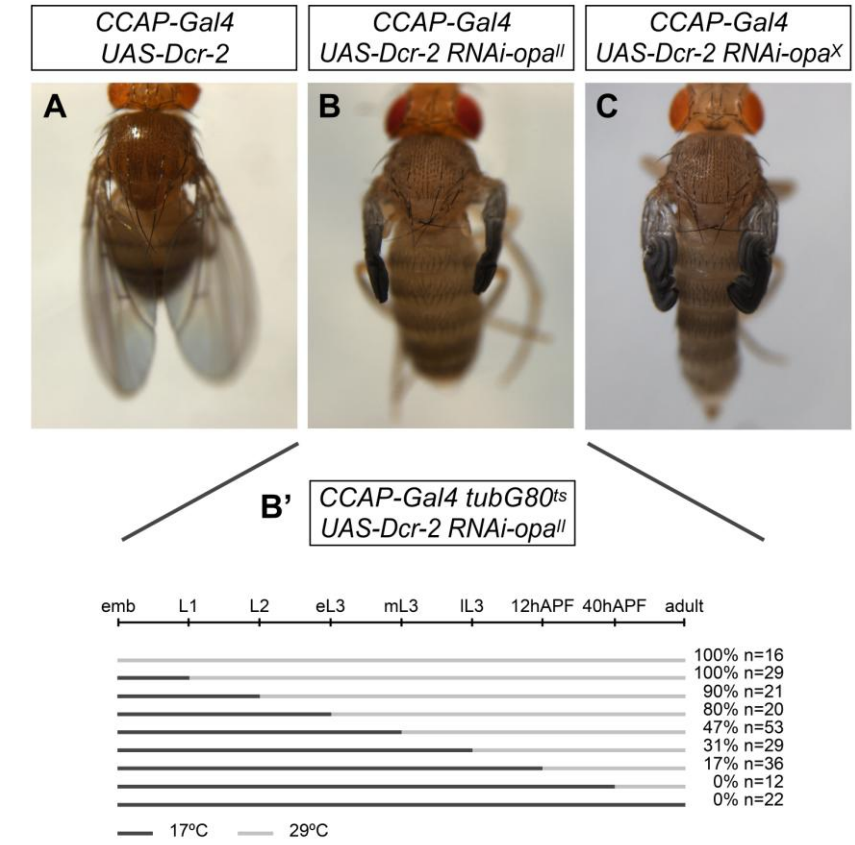
3 - Downregulation of *opa* expression in the CCAP neurons alters postecdysis behavior

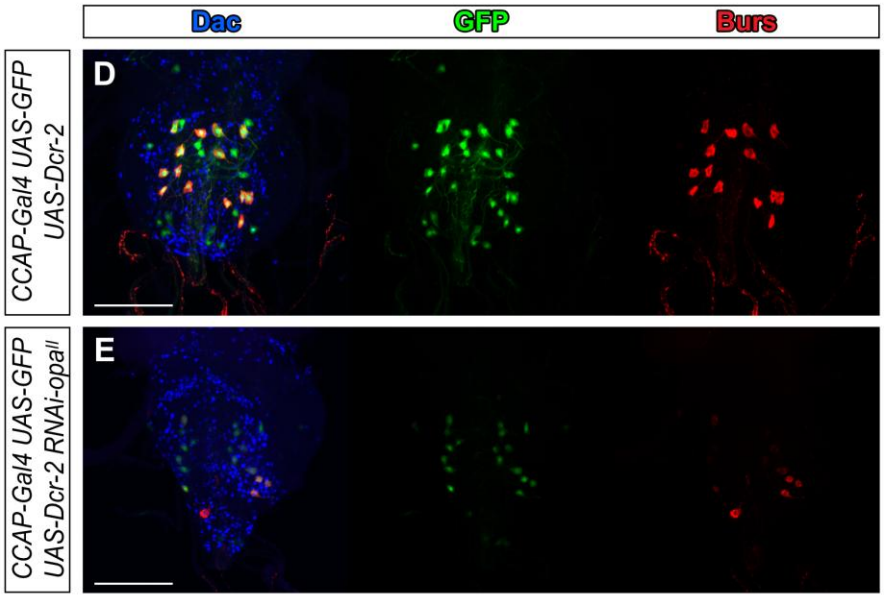
Once out of the pupal case, flies expand their wings within a period of 30 min. The cuticle of the thorax also expands, and the position of the two posterior bristles is a readout of this expansion. First these bristles cross each other in a pharate adult, and then during the posteclosion period, as a consequence of thorax stretching, they set themselves parallel to each other. The sclerotization and darkening of the cuticle takes place during the first 3h after eclosion. All these characteristics can be seen on our 3h aged *CCAP-Gal4 UAS-Dcr-2* control flies (**Figure 29A**). In order to figure out the role of *opa* during the posteclosion, we expressed ectopically the *RNAi-opa^{II}* in the CCAP cells through development. We noticed, in adult flies with 3h of age, a lack of wing extension and thorax expansion, evidenced by the position of the posterior thorax bristles, while the cuticle fails to harden and pigmentation is

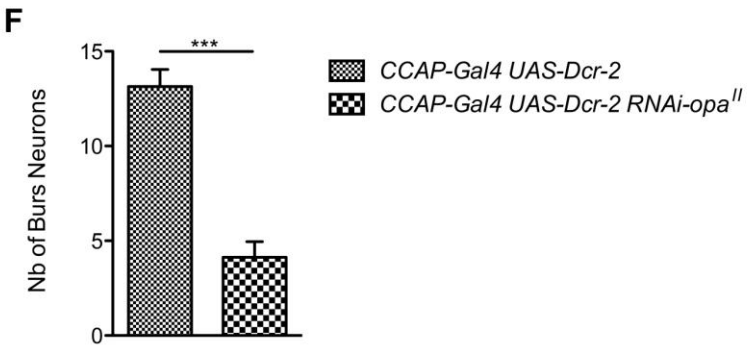
absent (**Figure 29B**). No lethality is associated with this genotype and all flies reach adulthood. Thus, to explore whether *opa* is required during a particular time during development we temporally controlled the *RNAi-opa*^{II} expression using the Gal80ts tool, an inhibitor of Gal4 function. In this way, we could express the *RNAi-opa*^{II} at different moments until eclosion and scored the percentage of adult flies presenting a phenotype. We noticed that the earlier *opa* function is inhibited the higher is the probability to have a fly presenting a posteclosion defect (**Figure 29B'**). In other words, Opa does not seem to be required in a particular developmental step, but rather continuously. Similar results were obtained using the *RNAi-opa*^{X} line (**Figure 29C** and not shown).

In newly eclosed wild type adult flies, the metathoracic and abdominal ganglia is composed of 14 neurons expressing *Burs* (Peabody et al., 2008; Lee et al., 2013). *Burs*-expressing neurons correspond to CCAP motoneurons and express the Dachshund (Dac) maker (Veverytsa and Allan, 2011). Similar features are found in our control *CCAP-Gal4 UAS-GFP UAS-Dcr-2* flies (**Figure 29D**). When we examined the VNC of *CCAP-Gal4 UAS GFP UAS-Dcr-2 RNAi-opa*^{II} flies we noticed an alteration in the driver expression due to the reduction of the GFP signal. We also observed a decrease in the number of the *Burs*-expressing neurons (**Figure 29E**). Statistical analysis (**Figure 29F**) revealed a 4-fold decrease of the *Burs*-expressing neurons in this condition compared to control. This result indicates an alteration in the execution of the postecdysis sequence in the adult flies when *opa* expression is reduced in the CCAP cells (**Figure 29B** and **C**).

Figure 29. Lack of *opa* expression leads to defects in the postecdysis behavior. (A) Control condition, corresponding to the ectopic expression of *Dcr-2* in the CCAP neurons, shows a normal postecdysis sequence. **(B,B')** Reduction of *opa* expression specifically in the CCAP neurons with *UAS-Dcr-2,RNAi-opa*^{II} leads to adult flies which do not inflate wings, fail to stretch their thorax and neither show a sclerotized nor melanized cuticle (B) (100%, n=150). The use of *tubG80*^{ts} permits to control temporally the ectopic expression *RNAi-opa*^{II} by a switch of temperature from 17°C (permissive temperature) to 29°C (restrictive temperature). Change of temperature at different developmental times does not reveal a specific requirement of Opa in the CCAP neurons (B'). **(C)** Reduction of *opa* expression specifically in the CCAP neurons by expressing another *RNAi-opa*, referred as *RNAi-opa*^{X}, together with *UAS-Dcr-2* (100%, n=120) shows the same defects that in (B). The pictures of adult flies in (A), (B) and (C) have been taken 3h after eclosion. **(D)** VNC control expressing *Dcr-2* in the CCAP cells. The expression of *CCAP-Gal4* and the immunostaining to Burs and Dac are shown. **(E)** Reduction of *opa* expression in the CCAP neurons leads to a decrease of *CCAP-Gal4* and *Burs* expression. VNC shown in (D) and (E) have been dissected from 0-1h adult flies. Scale bar: 100 µm. **(F)** Statistical analysis. For *CCAP-Gal4 UAS-Dcr-2*, n = 7; mean of number of neurons expressing *Burs* per VNC = 13,1 (± SEM 0,3). For *CCAP-Gal4 UAS-Dcr-2 RNAi-opa*^{II}, n = 8; mean of number of neurons expressing *Burs* per VNC = 4,1 (± SEM 0,3). **** p-value < 0.0001.


CCAP-Gal4
UAS-Dcr-2
CCAP-Gal4
UAS-Dcr-2 RNAi-opaII
CCAP-Gal4
UAS-Dcr-2 RNAi-opaX
A
B
C
B'
CCAP-Gal4 tubG80ts
UAS-Dcr-2 RNAi-opaII
emb
L1
L2
eL3
mL3
lL3
12hAPF
40hAPF
adult
100% n=16
100% n=29
90% n=21
80% n=20
47% n=53
31% n=29
17% n=36
0% n=12
0% n=22
17ºC
29ºC


Dac
GFP
Burs
CCAP-Gal4 UAS-GFP
UAS-Dcr-2
D
CCAP-Gal4 UAS-GFP
UAS-Dcr-2 RNAi-opaII
E


F
***
Nb of Burs Neurons
15
10
5
0
CCAP-Gal4 UAS-Dcr-2
CCAP-Gal4 UAS-Dcr-2 RNAi-opaII

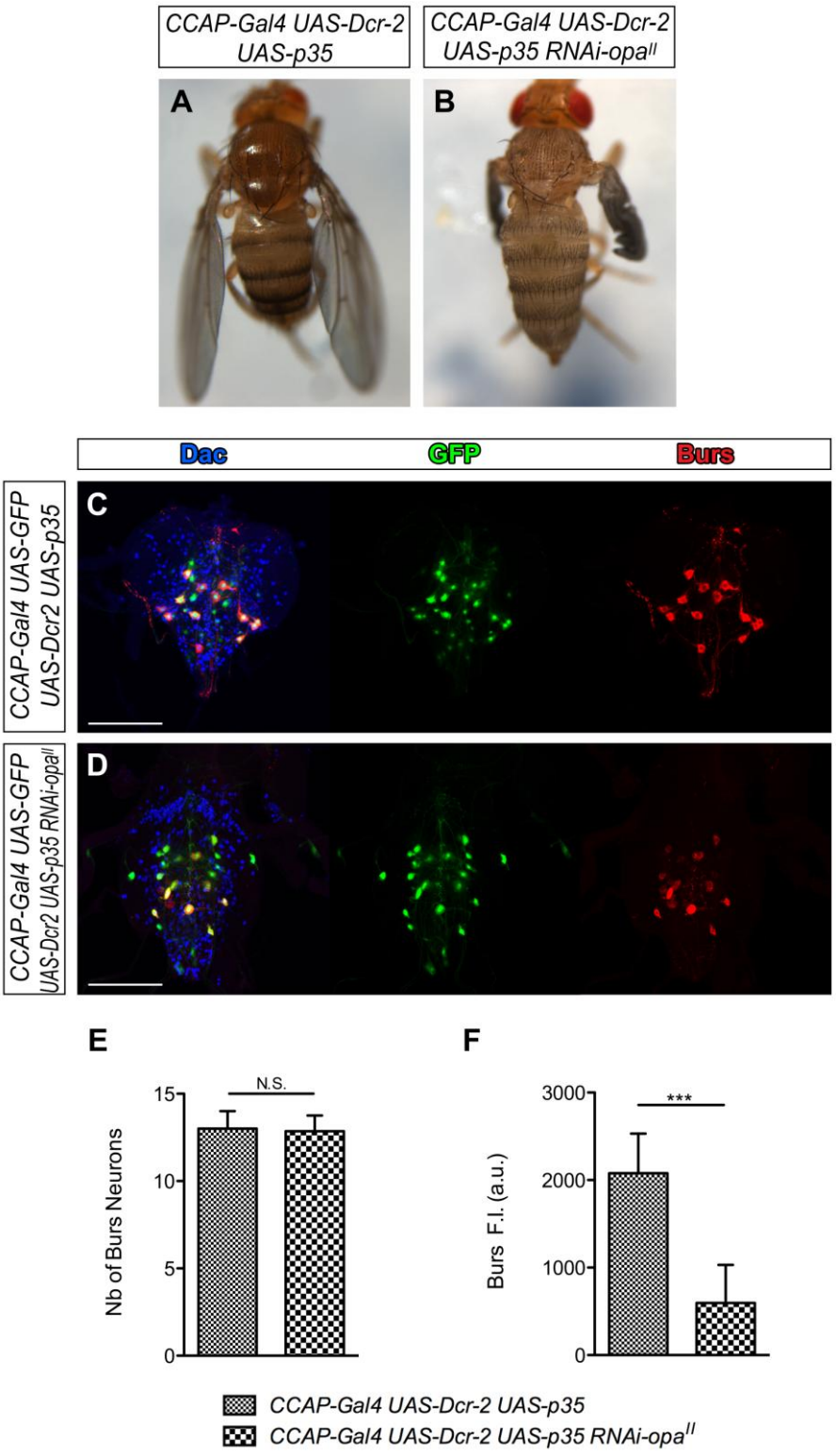
4 - Opa acts as a survival factor of the CCAP neurons and is a positive regulator of *Burs* expression

To explain how Opa affects *CCAP* and *Burs* expression, we considered two hypotheses, although not exclusive to each other. On the one hand, Opa could positively regulate *CCAP* and *Burs* expression; on the other hand, the decrease of signal of these neuropeptides could be consequence of CCAP cells death. To discriminate between these two possibilities, we used the cell death inhibitor *p35*. The genotype of our control flies is *CCAP-Gal4 UAS-Dcr-2 UAS-p35*. As presented in **Figure 30A**, posteclosion behavior occurs normally in these individuals. When we simultaneously block apoptosis and decrease *opa* expression level in flies *CCAP-Gal4 UAS-Dcr-2 UAS-p35 RNAi-opa^{II}* we were not able to see a rescue of the adult phenotype (**Figure 30B**).

CCAP cell death inhibition has no consequences either in the number of CCAP cells or in the expression of *CCAP* or *Burs* in a recently born adult (**Figure 30C**) (Vanden Broeck et al., 2013). But blocking apoptosis when *opa* is downregulated in *CCAP-Gal4 UAS-GFP UAS-Dcr-2 UAS-p35 RNAi-opa^{II}* flies (**Figure 30D**), the number of CCAP cells is similar to control (**Figure 30C**). The quantification is presented in **Figure 30E**. This result indicates that CCAP neurons enter in apoptosis when *opa* function is removed. Interestingly, we noticed that *Burs* expression in these brains (**Figure 30D**) is lower than in the control situation (**Figure 30C**). Quantification analysis reveals a 4-fold difference (**Figure 30F**). These results indicate that Opa, not only acts on maintaining CCAP viability, but also regulates *Burs* expression. Therefore, we

Figure 30. Opa regulates CCAP survival and is a positive regulator of Burs expression. (A) Ectopic expression of *p35* to block cell death together with *Dcr-2* does not affect normal postecdysial behavior. **(B)** Simultaneous ectopic expression of *UAS-p35 UAS-Dcr-2 RNAi-opa^{II}* in the CCAP neurons does not rescue the posteclosion phenotype induced by the lack of *opa* expression shown in Figure 29B (100%, n=60). The pictures shown in (A) and (B) of adult flies have been taken in 3h after eclosion flies. **(C)** VNC of control flies that express simultaneously *UAS-Dcr-2* and *UAS-p35* in the CCAP neurons. Inhibition of CCAP cell death has no effect on either CCAP cell number or *CCAP-Gal4* or *Burs* expression. **(D)** VNC of flies that express *UAS-p35 UAS-Dcr-2 RNAi-opa^{II}* ectopically in the CCAP cells of the VNC show similar CCAP cell number than control flies **(C).** However, the expression of *Burs* significantly decreased compared to control. VNC in (**C**) and (**D**) have been dissected from 0-1h adult flies. Scale bar: 100 µm. **(E,F)** Statistical analysis for *CCAP-Gal4 UAS-Dcr-2 UAS-p35* flies (**E**), n = 7; mean of number of neurons expressing *Burs* per individual VNC =13,0 (SEM ± 0,4) and for *CCAP-Gal4 UAS-Dcr-2 UAS-p35 RNAi-opa^{II}* flies (E), n = 7; mean of number of neurons expressing *Burs* in each VNC =12,9 (SEM ± 0,3). No significant difference (N. S.) means p-values≥ = 0.05. (F) Statistical analysis for *CCAP-Gal4 UAS-Dcr-2 UAS-p35* flies, n = 88 cells; mean of Burs Fluorescent Intensity (F. I) is in arbitrary units (a. u) per cell = 2079 (SEM ± 48). For *CCAP-Gal4 UAS-Dcr-2 UAS-p35 RNAi-opa^{II}* flies, n = 85 cells; mean of Burs F. I in a. u = 596 (SEM ± 47) per cell. **** p-value < 0.0001.

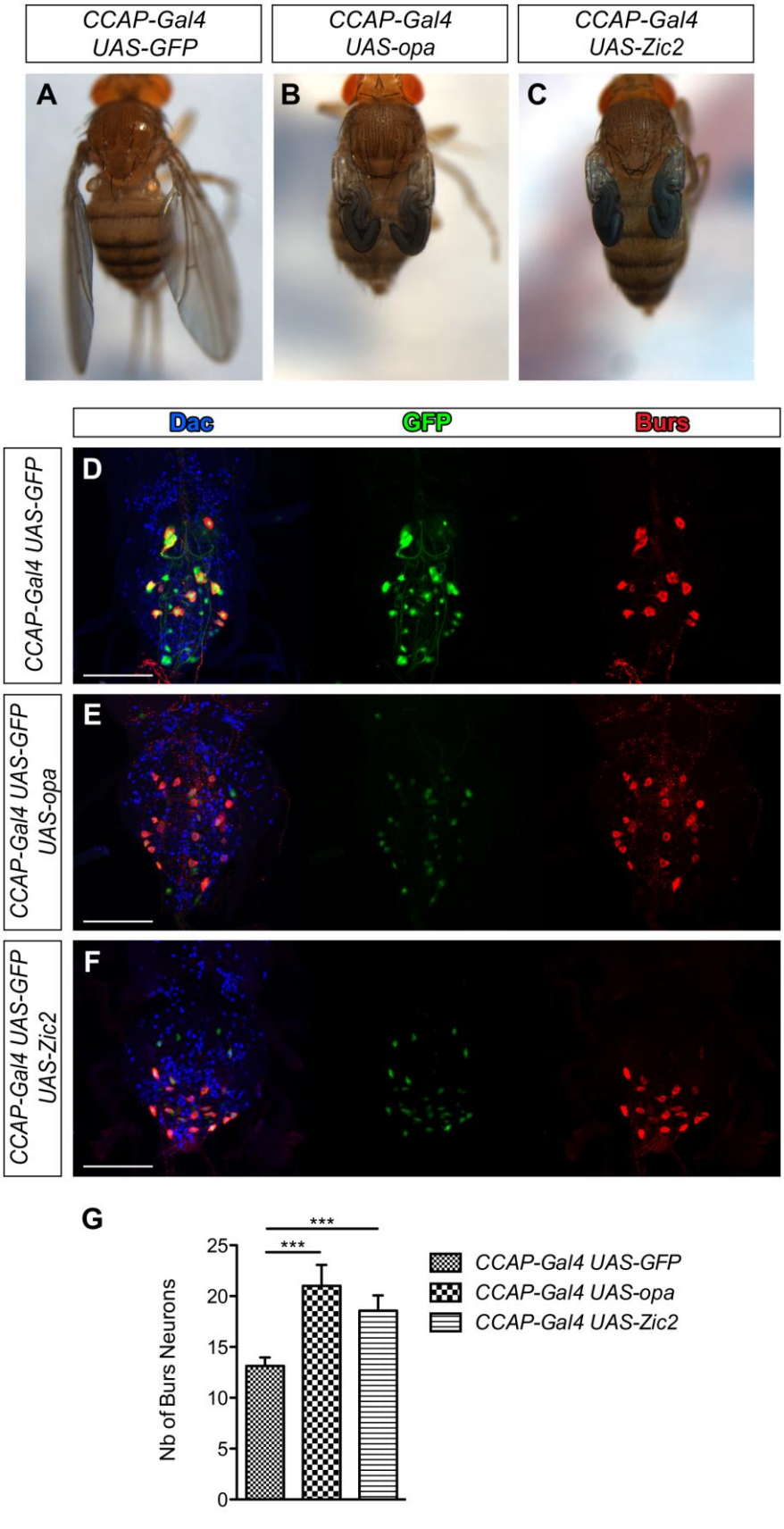
propose that the lack of phenotypic rescue when *opa* function is removed and simultaneously cell death is blocked in CCAP (**Figure 30B**) is due to a reduction of *Burs* expression.



5 - Ectopic expression of *opa* or *Zic*2 leads to adult, pupal and larval ecdysis defects by missexpressing *Burs*

Although it is clear that a correct ecdysis requires *opa* expression, we next wondered whether an excess of *opa* expression in the CCAP cells could also disturb the ecdysis sequence. All *CCAP-Gal4 UAS-opa* flies tested reached adulthood, but when compared to control flies (**Figure 31A**) they present characteristic features of alterations in the postecdysis sequence (**Figure 31B**). They exhibit wings completely fold and a lack of thorax extension, cuticle sclerotization does not occur properly as evidenced by a matte aspect, despite that melanization is normal. Similar postecdysis defects were obtained when expressing ectopically *Zic2* in the CCAP neurons (**Figure 31C**), although the penetrance of the phenotype is incomplete: 15% show a strong phenotype, another 15% a mild phenotype and the remaining 70% show a normal morphology (not shown). In control adult CNS, expression of *Burs* is restricted to the CCAP motoneurons (Dac+) and is absent from the CCAP interneurons (Dac-) (Luan et al., 2006). Thus, in neuromeres encompassing T3 and abdominal segments *Burs* is expressed in 14 CCAP of a total of 32 CCAP cells (**Figure 31D**). The total number of CCAP cells in adult brains of flies overexpressing *opa* in the entire CCAP population is similar to controls. However, the neuropeptides expressions change in an opposite way. Indeed *CCAP* expression decreases while *Burs* is not only detected in the CCAP motoneurons but also in the CCAP interneurons (**Figure 31E**). Soma of both cell types is similar but approximately halved the size of motoneurons soma control. CNS of flies expressing ectopically *Zic2* in the CCAP cells (with a strong posteclosion phenotype) presents the same characteristics (**Figure 31F**). Statistical analysis (**Figure 31G**) indicates that

Figure 31. Overexpression *opa* or ectopic expression of *Zic2* in the CCAP neurons affects postecdysis behavior by activating ectopically *Burs* expression in the CCAP interneurons. (A) Adult *CCAP-Gal4 UAS-GFP* flies used as control. **(B)** Overexpression of *opa* in the CCAP neurons causes defects in adult ecdysis: the wings do not expand, the thorax fails to stretch, the cuticle is matte but the pigmentation is similar to control (100%, n=83). **(C)** Ectopic expression of *Zic2* is also able to alter the postecdysis behavior but with less frequency (30%, n=93). In (A-C) pictures were taken from 3h adults. **(D)** Adult VNC control expressing *CCAP-Gal4 UAS-GFP* and stained for Burs and Dac. **(E)** Overexpression of *opa* in the CCAP neurons (same genotype as in (B)) leads to a general decrease of *CCAP* expression, but also an ectopic expression of *Burs* in the CCAP interneurons. **(F)** Ectopic expression of *Zic2* in the CCAP neurons (VNC are dissected from flies with the strongest phenotype of the genotype presented in (C)) shows the same alteration as in (E). VNC in (E) and (F) were dissected from 0-1h adults. Scale bar: 100 µm. **(G)** Statistical analysis. For *CCAP-Gal4 UAS-GFP*, n = 8; mean of number of neurons expressing *Burs* per VNC = 13,1 (SEM ± 0,3). For *CCAP-Gal4 UAS-opa*, n = 7; mean of number of neurons expressing *Burs* per VNC = 21,0 (SEM ± 0,8). For *CCAP-Gal4 UAS-Zic2*, n = 7; mean of number of neurons expressing *Burs* per VNC = 18,6 (SEM ± 0,6). **** p-value < 0.0001.


CCAP-Gal4 UAS-GFP
CCAP-Gal4 UAS-opa
CCAP-Gal4 UAS-Zic2
A
B
C
Dac
GFP
Burs
CCAP-Gal4 UAS-GFP
D
CCAP-Gal4 UAS-GFP UAS-opa
E
CCAP-Gal4 UAS-GFP UAS-Zic2
F
G
***
***
Nb of Burs Neurons
25
20
15
10
5
0
CCAP-Gal4 UAS-GFP
CCAP-Gal4 UAS-opa
CCAP-Gal4 UAS-Zic2

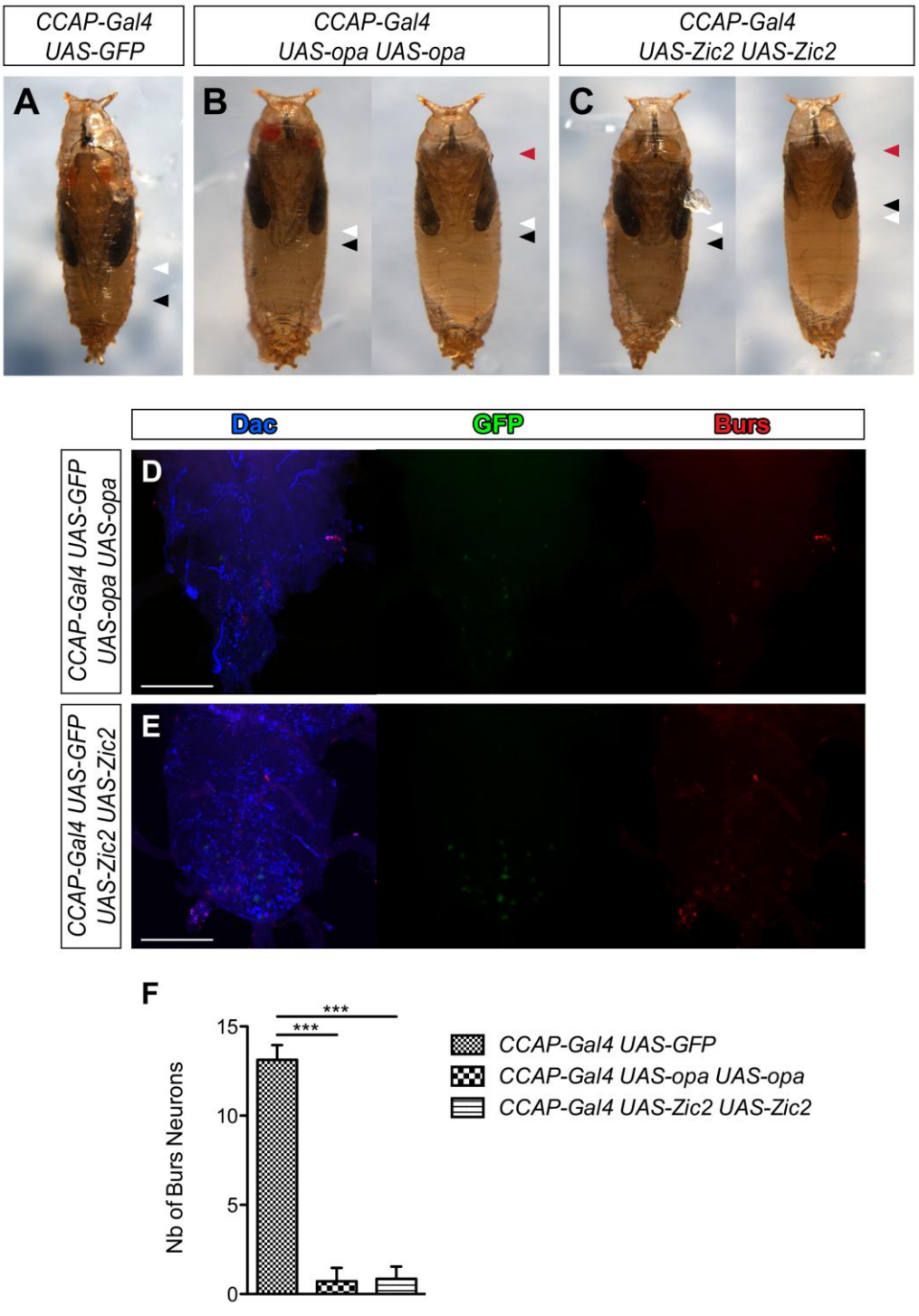
significantly in both genotypes more CCAP neurons express *Burs* than in controls. All together, these results indicate that overexpression of *opa* or ectopic expression of *Zic2* negatively represses *CCAP* and conversely positively regulates *Burs* expression. Given that the neuropeptide CCAP is not implicated in the adult ecdysis (Lahr et al., 2012) we propose that the postecdysis phenotype is due to an excess of Burs in the CCAP interneurons.

A premature lethality could be observed when two copies of *UAS-opa* or *UAS-Zic2* were expressed under the control of *CCAP-Gal4*. Indeed these individuals die in metamorphosed pupa. Compared to control pupae (**Figure 32A**) these pharates do not extend their legs and wings properly (**Figure 32B** and **C**, black and white arrows respectively). In the most severe cases, the head does not evert and remains trapped in the thorax (**Figure 32B** and **C**, red arrow). These defects are characteristic of a failure in the pupal ecdysis and are reminiscent of the pupal phenotype when the CCAP are genetically ablated (Park et al., 2003). A first morphological observation is that the CNS of these pharates is more compact than the control brain (2X) and the general brain shape seems to be closer to a L3 brain than to an adult brain (not shown). *CCAP* and *Burs* expressions were almost absent (**Figure 32D** and **E**). Quantification of the number of *Burs*-expressing neurons for both genotypes compared to control is shown in **Figure 32F**. However, it remains to be determined whether the reduction of *Burs* expression is a consequence of CCAP cell death.

Next, we wanted to test whether the capacity of Opa to activate ectopically *Burs* expression is specific to the CCAP neurons or cell type independent. To this aim, we first expressed *opa* under the control of *elav-Gal4*, expressed in all neuronal cells (Robinow and White, 1991). As these individuals die during embryogenesis, we used *tubG80ts* to initiate ectopic expression during larval stage. These flies do not reach adulthood and die during the metamorphosis or

Figure 32. *opa* or *Zic2* gain-of-function affects pupal ecdysis. (A) A pharate adult *CCAP-Gal4 UAS-GFP* used as a control. **(B,C)** Representative examples of pharate adults upon expression of two copies of *UAS-opa* (B) or *UAS-Zic2* (C) showing abnormal pupal ecdysis based on the lack of wing and leg extension and sometimes on the lack of head eversion. The distal part of the wing and legs are marked in (A), (B) and (C) by white and black arrowheads respectively and the red arrowheads point out the head trapped into the thorax. (For both genotypes, 100% of the animals observed show pupal defects with various grade of severity. For (B) n = 52 and for (C) n = 70). **(D,E)** Overexpression of two copies of *UAS-opa* (D) or ectopic expression of two copies of *UAS-Zic2* (E) in the CCAP neurons decreases *CCAP* and *Burs* expression in the pharate VNC. Scale bar: 100 µm. **(F)** Statistical analysis for *CCAP-Gal4 UAS-GFP*, n = 8; mean of number of neurons expressing *Burs* per VNC = 13,1 (SEM ± 0,3), for *CCAP-Gal4 UAS-opa*, n = 7; mean of number of neurons expressing *Burs* per VNC = 0,7 (SEM ± 0,3), and for *CCAP-Gal4 UAS-Zic2 UAS-Zic2*, n = 7; mean of number of neurons expressing *Burs* per VNC = 0,8 (SEM ± 0,3). **** p-value < 0.0001.

even at an earlier stage (in conditions where *opa* is expressed ectopically 4 days long (**Figure 33A** and not shown). Larvae CNS of this genotype show an evident generalized ectopic expression of *Burs* (**Figure 33B**). Indeed, under this condition, *Burs* ectopic expression is detected in several ventral VNC cells in the thoracic and the first abdominal segments



(**Figure 33B** and not shown). Wild type *Burs* expression in a larval VNC is shown in **Figure 33C**. Because expressing ectopically *opa* during a longer time period is lethal, we could not test whether *Burs* could be ectopically expressed in more neurons increasing the time of *opa* ectopic expression. We also expressed *opa* under the control of 3*6Y-Gal4*, a peptidergic line (O'Brien and Taghert, 1998). Here, we also used *tubG80ts* to overcome the embryonic lethality associated with this gain-of-function expression. After 3 days of *opa* ectopic expression, animals die during the early stages of metamorphosis or during larval stage (**Figure 33D** and not shown). These larvae display a typical molting defect: they present double set of mouth hooks (**Figure 33E**, arrow) while wild type larvae have a unique set of mouth hooks (**Figure 33F**). Dissecting larvae CNS, we found that in the lateral VNC sides, Opa is able to ectopically activate *Burs* in several, but not all, *36Y-Gal4*-expressing neurons (**Figure 33G**).

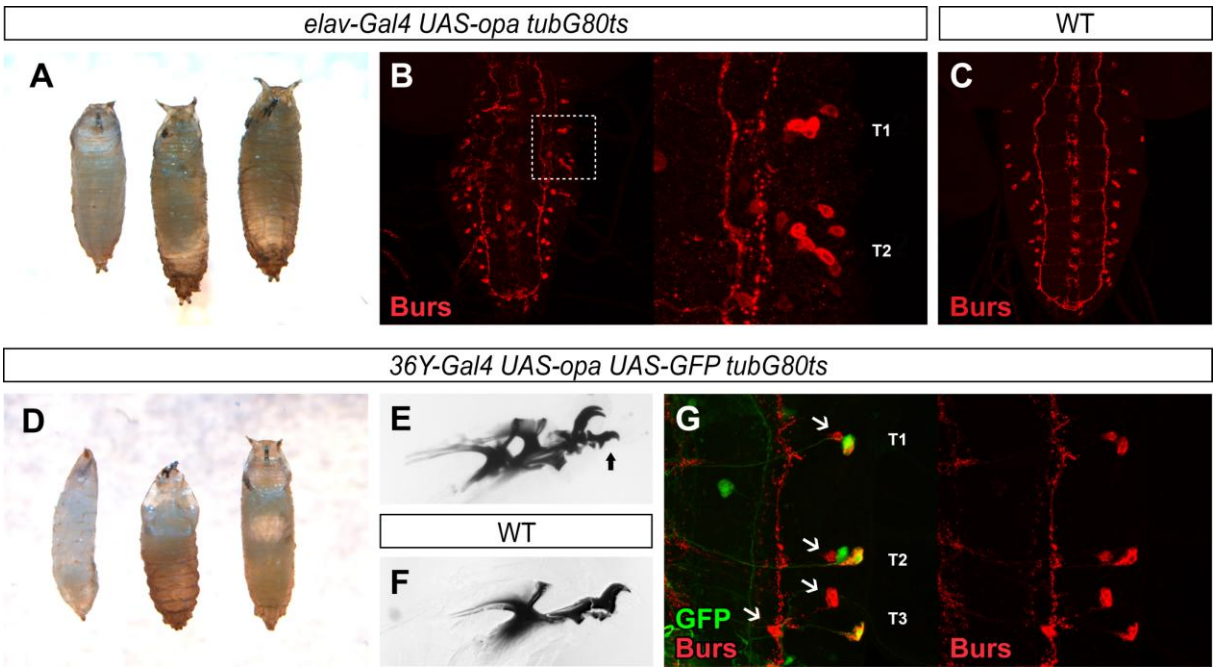


Figure 33. Opa is able to activate *Burs* expression in no-CCAP neurons. (A) Ectopic expression of *opa* under the control of *elav-Gal4* leads to early pupal lethality. **(B)** Larval CNS from the previous genotype showing an ectopic expression of *Burs* in a large amount of neurons, especially in lateral neurons of the VNC. **(C)** WT expression of *Burs* in the VNC of a L3 larva. At this stage, hemineuromeres T1 and T2 express *Burs* in one neuron and hemineuromeres T3-A7 express *Burs* in two cells. **(D)** Ectopic expression of *opa* under the control of *36Y-Gal4* also leads to early pupal lethality. **(E)** In larvae of similar genotype, the L2 mouth parts (arrow) do not detach from the L3 mouth hooks. **(F)** WT mouth hooks. **(G)** Ectopic expression of *Burs* can be observed in a subset of *36-Y>opa*-expressing cells in the lateral VNC. The arrowheads indicate the endogenous expression of *Burs*. (B), (C) and (G) correspond to maximum projection of stacks.

6 - Opa does not control CCAP motoneurons axon guidance during the larval stage

In the larva, CCAP motoneurons project their axons towards the ventral midline and they have been described to innervate the ventrolateral muscle M12. The M13, which is more ventral than the M12 and parallel to it, is also sometimes innervated (Hodge et al., 2005). We wanted to determine whether Opa could be involved in CCAP motoneurons axon guidance during larval stage. CCAP motoneurons end up innervating the M12 all along the fiber allowing an easy identification of the muscle (**Figure 34A**). We next downregulated *opa* expression in the CCAP neurons (which are preserved from death by expressing ectopically *p35*), and examined CCAP axonal behavior. Under this condition, we could see that, in each muscular hemisegment, the muscles M12/M13 are apparently correctly innervated (**Figure 34B**). A similar result was obtained upon *opa* overexpression in the CCAP (**Figure 34C**). These results suggest that Opa has no effect on larval CCAP motoneurons pathfinding.

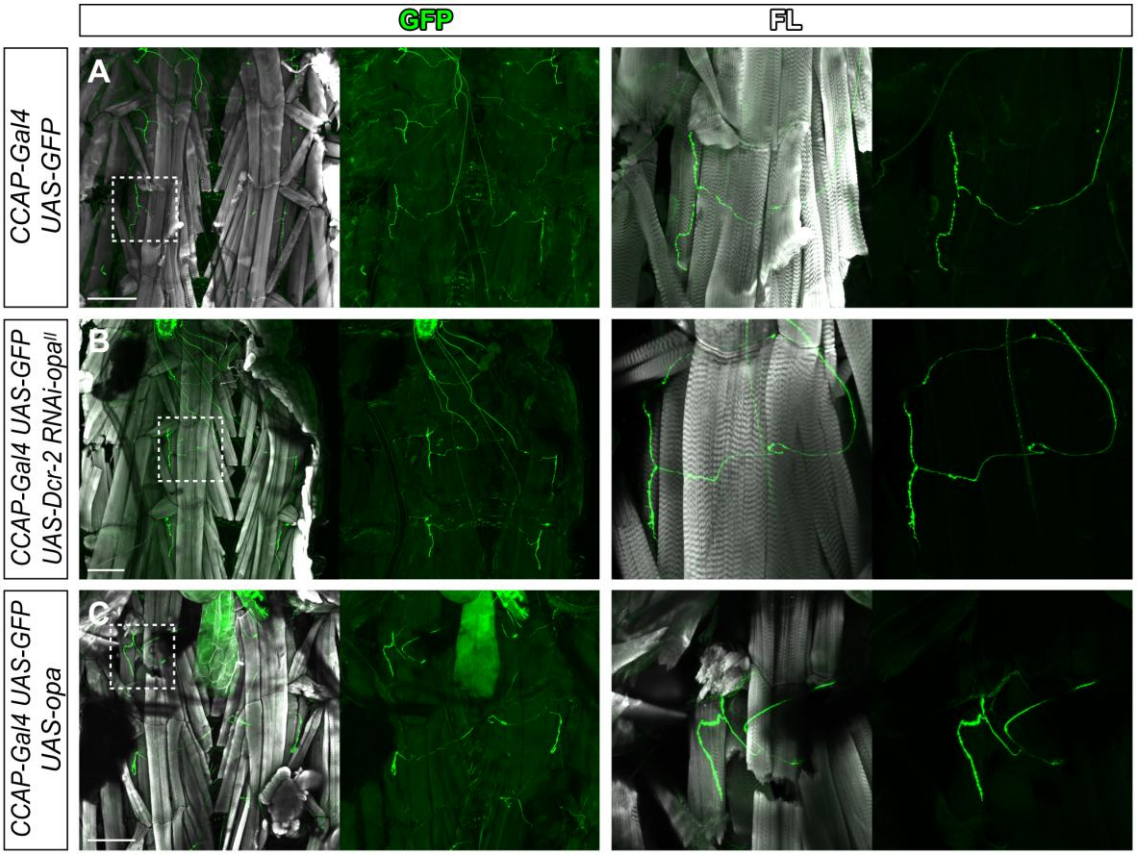


Figure 34. Projection of CCAP motoneurons to larval bodywall muscles upon *opa* missexpression. *CCAP-Gal4 UAS-GFP* filleted larva showing innervations of the muscles M12/M13 **(A)**. Similar innervations pattern is observed in *CCAP-Gal4 UAS-GFP UAS-RNAi-opa^{II} UAS-Dcr2 UAS-p35* **(B)** and in *CCAP-Gal4 UAS-GFP UAS-opa* filleted larvae **(C)**. Phalloidin (FL) marks the filamentous actin. Scale bar: 200 µm.

7 - Observation of the axonal projections of CCAP motoneurons into adult abdominal muscles

During pupal stage CCAP axons suffer a remodeling (Zhao et al., 2008; Gu et al., 2013) but the innervation target muscles in the adult are not known. In addition, during metamorphosis, although most of the larvae muscles are hydrolyzed and adult muscles are formed *de novo*, some larval muscles escape degradation and are maintained until the first hours of adulthood. It has been described that at 24h after eclosion, these muscles are not visible anymore and the fly abdomen presents its definitive muscular pattern (see **Figure 7** of the introduction; Kimura and Truman, 1990). Because of the timing of degradation, these larva-inherited adult muscles constitute ideal candidates to be innervated by the CCAP motoneurons.

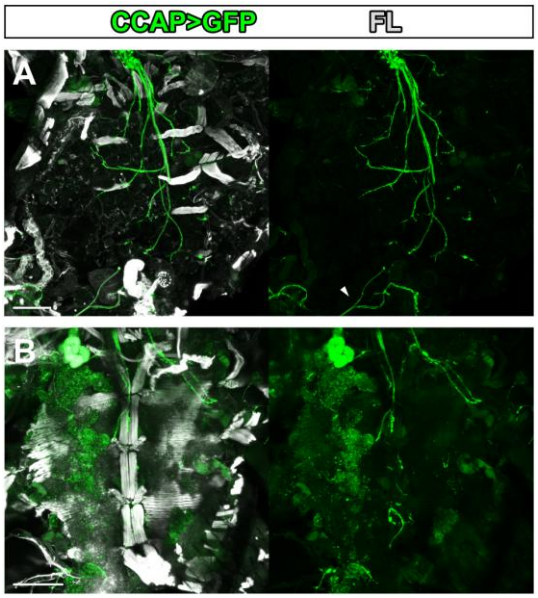


Figure 35. Timing correlation between larva-inherited adult muscles degradation and CCAP axons fragmentation. (A) Adult ventral abdomen at 0h showing an extensive arborization of the CCAP neurons from the VNC. The arrowhead points out CCAP axons coming from the posterior extremity. **(B)** Adult ventral abdomen at 24h revealing CCAP axonal degeneration. Anterior is towards the top. Scale bar: 200 µm.

First, we observed the general axonal arborization of CCAP motoneurons in adult flies at 0h of age. At this time, CCAP motoneurons of the VNC present a more complex axonal organization than in the larva due to axonal ramifications (**Figure 35A**). Interestingly, we could also detect CCAP axons from the most posterior part of the adult abdomen, which do not seem to be related to the CCAP neurons of the VNC (**Figure 35A**, arrow and also seen later in **Figure 37**). 24h after eclosion, CCAP neurons present fragmented axons (**Figure 35B**). Therefore it appears that the timing of axonal degeneration coincides with the degradation of the doomed muscles during the first hours of adulthood.

On the ventral side, the larva-inherited adult muscles are easily identifiable by their shapes: in the A2 segments the pleural external longitudinal muscles (PELM) are composed of 4 fibers describing a double “V” shape, called flanking the abdominal muscles of the midline. We could see that these PELM are highly innervated by CCAP axons coming from the VNC at

0h adults (**Figure 36A**). At this stage, the pleural internal transverse muscles (PITM) in all intersegments present elongated fibers perpendicular to the midline, which extend from the ventral midline to the lateral sides. We could see that these muscles are often innervated by CCAP axons. However, we found that at the proximity of these muscles a CCAP body cell is detected. Interestingly, *opa* is expressed in these peripheral neurons, which are also bipolar and seem to be in contact with the CCAP neurons from the VNC (**Figure 36B** and **C**). However, as all the axons of the CCAP neurons are identified in the same way we cannot ensure where the contact between all these different CCAP neurons population occurs. Note that for each PITM muscle observed either one CCAP body cell or none is detected close by.

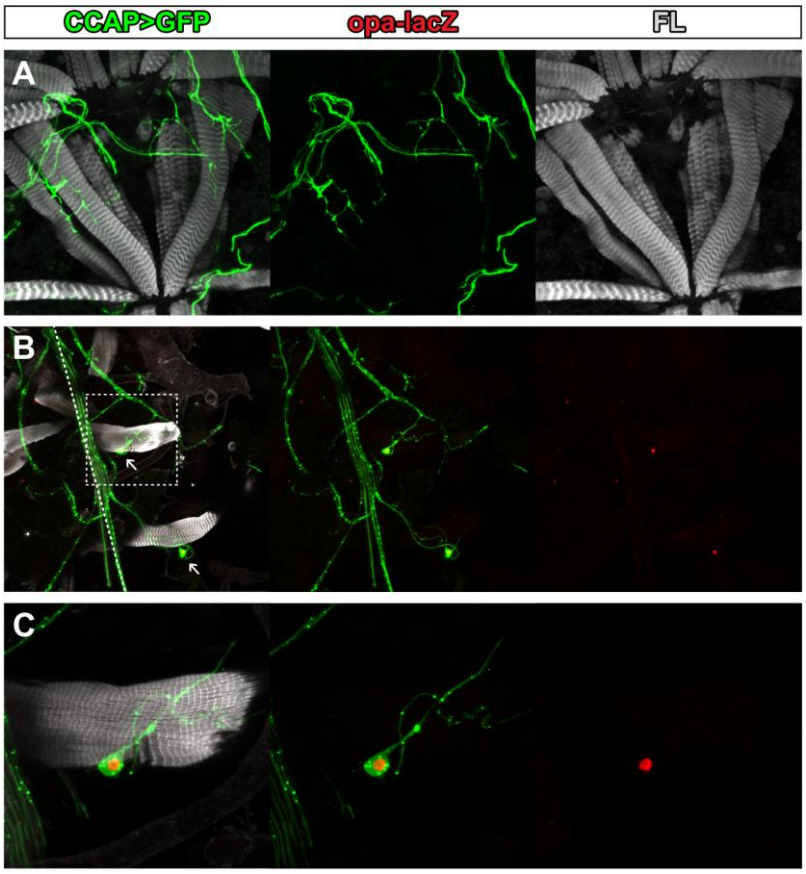


Figure 36. The larva-inherited adult muscles are innervated by two different populations of CCAP neurons. (A) PELM of the A2 segment showing rich innervations by the CCAP neurons from the CNS. **(B)** PITM at the A2/A3 and A3/A4 borders also show innervations by CCAP neurons. However, they are respectively innervated by a peripheral bipolar CCAP neurons expressing *opa* (arrows). These neurons appear to be physically in contact with axons from CNS CCAP neurons. The dashed line indicates the ventral midline. **(C)** A zoom from the region marks by a square in (B). Images correspond to a maximal projection of stacks. Anterior is towards the top.

8 - Identification of CCAP neurons outside the CNS

We would like to close this second chapter by reporting the existence of other CCAP neurons outside the CNS (**Figure 37**). It has been previously described that several CCAP neurons located on the lateral sides of the adult abdomen innervate the dorsal vessel (**Figure 37A**; Dulcis and Levine, 2003; Dulcis et al., 2004). We identified a further new CCAP cell populations located in the posterior part of the adult abdomen especially surrounding the rectal ampulla of both genders (**Figure 37B** and not shown). In female, the oviducts are also surrounded by a CCAP (**Figure 37C**). Another CCAP is also observed close to the spermatheca (**Figure 37D**). In male, the inner face of the ejaculatory bulb is highly innervated by a CCAP neuron (**Figure 37E**). Consistent with the previous observation that *opa* is expressed in the CCAP neurons of the CNS, and in the one innervating the larva-inherited adult muscles, we found that these peripheral CCAP neurons also express *opa* (**Figure 37**).

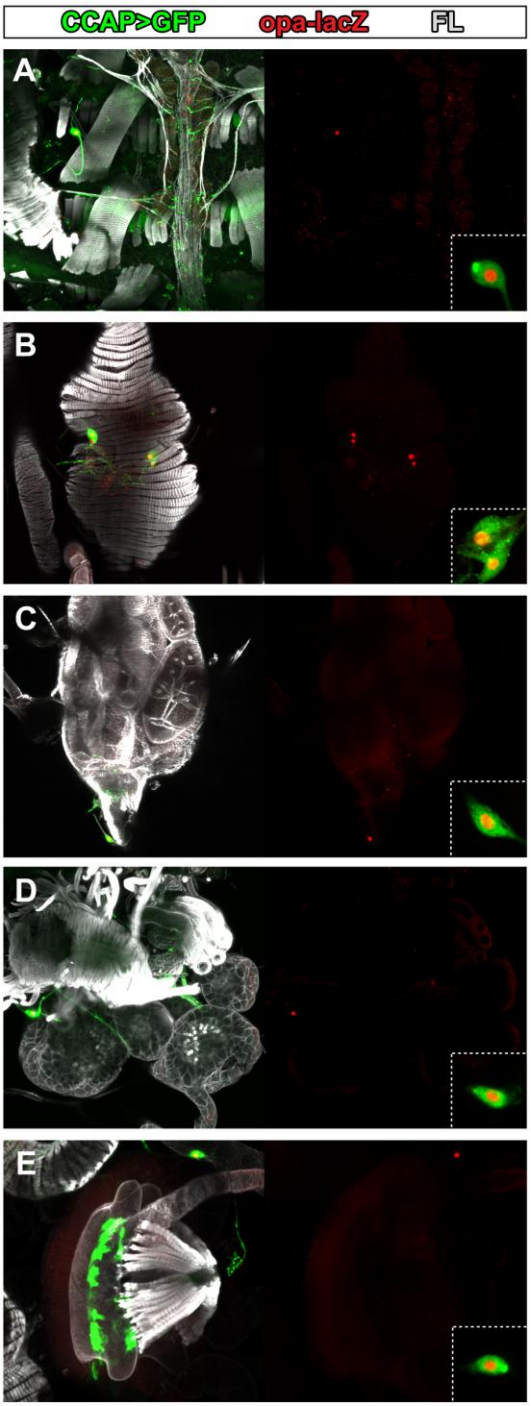


Figure 37. Existence for peripheral CCAP neurons expressing *opa*. CCAP neurons innervating the aorta **(A)** the rectal ampulla (here of a female) **(B)**, the oviduct **(C)**, the spermatheca **(D)** and the ejaculatory bulb **(E)**. All these neurons expressed *opa*. All images are maximum projections of stacks. The inserts showing *opa* expression in the CCAP correspond to a single section.

Discussion

1 - Opa as a marker of the CCAP neurons

In the second part of this Thesis we have studied the role of the transcription factor Opa during the posteclosion period. We found that *opa* is expressed, in the CNS, in all the CCAP cells, motoneurons and interneurons, from the embryonic stage 12 to the adult stage (**Figure 22** and **23**), indicating that *opa* is expressed throughout the lifetime of the CCAP. *opa* expression is not exclusive to the CCAP neurons but its expression is relatively limited. In the dorsal part of the VNC, *opa* expression is restricted to the CCAP cells (**Figure 24**); the rest of the *opa*-expressing cells are found mainly in the ventral part of the VNC. We identified some of these cells as peptidergic cells (**Figure 25**). So far, few markers have been identified to be expressed in the CCAP neurons: Dac, phosphorylated Mad (pMad) and *Ok6-Gal4* but their expression is limited to the CCAP motoneurons and they have the disadvantage to be expressed in many more neurons throughout the VNC. Thus, Opa seems to be an appropriate marker for the CCAP cells, both motoneurons and interneurons.

In the fly, most of the CCAP neurons start expressing *CCAP* since the embryogenesis. However, a subset of 12 posterior CCAP cells starts expressing the neuropeptide prior to the

metamorphosis, under the control of ecdysone signaling. These neurons are particularly interesting because they are sufficient for pupal ecdysis (Veverytsa and Allan, 2012). Based on lineage tracing experiment (since, as mentioned above, no specific markers were described to be expressed in the CCAP), it has been proposed that these neurons are generated during embryogenesis but achieve their terminal differentiation at pupariation (Veverytsa and Allan, 2012), similarly to what occurs in the sphinx moth *Manduca sexta* (Davis et al., 1993; Loi et al., 2001). The expression of *opa*, since the early life of all the CCAP neurons, allows to follow CCAP cells through development and therefore to confirm the prediction that the CCAP neurons that undergo a late terminal differentiation are generated early during development.

Different types of CCAP neurons are present in the abdomen of the adult fly whose function is to regulate dorsal vessel contractions by expressing the *CCAP* neuropeptide (Nichols et al., 1999; Dulcis and Levine, 2003; Dulcis et al., 2005). This role of modulator of heart contraction has been previously identified in *M. sexta* and the shore crab *Carcinus maenas* (Tublitz and Evans, 1986; Stangier et al., 1987) indicating a conservation of the *CCAP* function between species. In the locust *Locusta migratoria*, CCAP neurons stimulate the contractions of the oviducts and the spermathecas (da Silva and Lange, 2006). Also, in *L. migratoria* and *M. sexta*, the third neuropeptide expressed in the CCAP neurons, MIP, inhibits spontaneous muscle contractions of the gut and the oviducts (Schoofs et al., 1991; Blackburn et al., 1995). Although we have not tested whether these processes are conserved in the fly, we found that the muscles of the rectal ampolla, the oviduct and the spermatheca of *Drosophila* are also innervated by a new CCAP cells population (**Figure 37**). Besides, we have also identified new organs innervated by CCAP such as the larva-inherited adult muscles and the ejaculatory bulb (**Figure 36** and **37**). Interestingly, all these peripherical CCAP neurons express *opa* (**Figure 36** and **37**), indicating that Opa constitutes a general marker of the CCAP neurons.

2 - Opa prevents CCAP neuron from cell death

Drosophila CNS is constantly submitted to neuronal death (reviewed in Pinto-Teixeira et al., 2016) and particularly during embryogenesis and at the beginning of adulthood. Usually, a given NB generates the same lineage in all the neuromeres, and cell death is in charge to discard the unnecessary neurons depending on the neuromere. This phenomenon occurs

with the lineage of the NB 3-5. Indeed, it gives rise to one CCAP motoneuron and one CCAP interneuron, in each hemineuromere from SE1-A7, but motoneurons from SE1-T2 undergo programmed cell death as soon as they are born (Moris-Sanz et al., 2014). In has been shown that ectopic expression of the Hox gene *Ubx* is sufficient to prevent apoptosis in these cells. Also, it has been reported that Ubx and Abd-A prevent CCAP motoneurons death from the segment T3-A1 and A2-A7 respectively (Moris-Sanz et al., 2015). When CCAP neurons have accomplished their function after the eclosion, they progressively degenerate (Lee and al., 2013).

When *opa* is downregulated in the CCAP neurons, we observed a significant decrease in the number of the CCAP cells, of both motoneurons and interneurons (**Figure 29**). This number is recovered when apoptosis is blocked indicating that Opa acts as a survival factor of the CCAP neurons (**Figure 30**), which led us to propose that Opa acts as a CCAP survival factor. Control of the timing of *opa* downregulation did not reveal a requirement of Opa at a particular developmental stage, meaning that CCAP neurons need constantly Opa expression to be maintained alive. Interestingly, in the zebrafish *Dario rerio*, it has been shown that *Zic1* depletion induces cell death in the prospective diencephalon (Maurus and Harris, 2009) and knockdown of *Zic2* leads to cell death during neuronal differentiation (Luo et al., 2015). These results indicate a conservation of the *Zic* genes functions in preventing neuronal cell death.

In a wild type situation, it is known that few days after eclosion most of the CCAP neurons have entered apoptosis. Therefore, it would be interesting to test whether CCAP cell death is triggered by a diminution of *opa* expression during the first days of adulthood. An appropriate experiment to tackle this question would be to observe *opa* expression a few days after eclosion in flies were the CCAP apoptosis is blocked. Alternatively, it would be worth to overexpress *opa* in the CCAP and observe whether several days after eclosion CCAP neurons are still present.

3 - Opa, an activator of *Burs*

In addition to the function of Opa in the CCAP cell viability we found that Opa acts as a positive regulator of *Burs* expression. In thoracic and abdominal segments of a wild type adult, *Burs* is strictly expressed in the CCAP motoneurons T3-A7 (Luan et al., 2006). When,

opa function is reduced in CCAP cells (and cell death prevented) a reduction of the *Burs*-expressing cell is observed in newly formed adult CNS compared to control (**Figure 30**). Conversely, when *opa* is overexpressed in the CCAP cells, *Burs*, in addition to be expressed in the motoneurons T3-A7, is detected in the most posterior motoneurons and in the interneurons (**Figure 31**). Interestingly, similar results, although weaker, have been obtained when expressing *Zic2* ectopically in the CCAP neurons (**Figure 31**) underlying a conserved mechanism of action throughout Zic proteins. To know whether an excess of *opa* expression is able to activate *Burs* expression uniquely in this particular cell type or not, we expressed ectopically *opa* under the control of a pan-neuronal line and of a peptidergic line. In both cases, we could see an ectopic *Burs* expression in non CCAP neurons (**Figure 33**), indicating that Opa is able to activate *Burs* expression in neurons different from the CCAP cell type identity. However, our experiments seem to indicate that Opa is not sufficient to activated *Burs* expression in all neurons. Because the ectopic expression of *opa* with *elav-Gal4* and *36Y-Gal4* is embryonic lethal we used *tubG80ts* to express *opa* during the postembryonic development. Under this condition, we saw that an important part of neurons, but not all, activate *Burs* expression. Maybe at this stage, some neurons are aged enough to not be competent to activate *Burs* expression. Alternatively it may be a matter of neuronal type; some may be more sensitive to respond to *opa* ectopic expression that others.

Burs expression evolves over time. Indeed, during larval stages it is expressed in the CCAP interneurons and its expression is turned off during the metamorphosis (Luan et al., 2006; Peabody et al., 2008; Vanden Broeck et al., 2013). The reason for this change has not been determined yet. Based on our results, it is likely that Opa activates *Burs* expression in these interneurons during early stages and a hypothetic repressor might start to be expressed at later stages to impede these neurons to continue expresseing *Burs*. Alternatively, the lack of *Burs* expression in the adult CCAP interneurons may be due to the lack of a coactivator.

It is more difficult to establish a regulation of *CCAP* by Opa. Indeed, if this Thesis we have used a *CCAP-Gal4 UAS-GFP* construct mainly as a way to identified the CCAP cells rather than monitoring the expression of the *CCAP* neuropeptide with the intensity of the *UAS-GFP*. Based on our results, it seems that reducing *opa* expression level, while blocking cell death, does not seem to have a consequence on *CCAP-Gal4 UAS-GFP* expression but no rigorous quantifications have been performed. Therefore, it would be appropriate to test CCAP expression using an anti-CCAP antibody. Nonetheless, it is clear enough that ectopic expression of *opa* is able to regulate negatively *CCAP-Gal4* expression in the CCAP cells. Also,

it would be interesting to test *CCAP* expression (through the use of a specific antibody) when *opa* is ectopically expressed in different neurons from the CCAP type.

To our knowledge no other study has been dedicated to understand the functional role of a transcription factor in the process of ecdysis mediated by *Burs* in arthropods. However, a recent screening, via dsRNA injection, looking for genes implicated in the metamorphosis of the red flour beetle, *Tribolium castaneum*, identified Opa as unique transcription factor candidate (Linz and Tomoyasu, 2015), indicating a possible conserved function of Opa in insects. There is no doubt that it would be very exciting to test whether *opa* is expressed not only in other insect species but also in other arthropod orders and test if the regulation of *Burs* by Opa is conserved.

4 - Opa and axon pathfinding

Larval muscles M12 and M13 are known to be innervated by the CCAP motoneurons (Hodge et al., 2005; Vomel and Wegener, 2007). Single cell labeling experiments have permitted to establish that CCAP axons cross the midline before exiting the VNC (Peabody et al., 2008; Karsai et al., 2013). We tested whether missregulation of *opa*, by downregulating its expression or overexpressing it, could alter CCAP pathfinding. However we did not find any evidence of an implication in the correct projection of CCAP axon during the larval stage. These observations contrast with the known role of *Zic2* in the retinal ganglion cell axon guidance to the optic chiasm (Herrera et al., 2003).

At the metamorphosis, under control of the insulin pathway (Gu et al., 2013) CCAP pruning completely change axonal projections (Zhao et al., 2008). We identified the target muscles of the CCAP neurons after metamorphosis: the muscles inherited from the larva, which have been described to be required during the postecdysis period (Kimura and Truman, 1990). The timing of degradation of these muscles is concomitant with the entrance in apoptosis of the CCAP neurons. Both events occur gradually during the first hours following eclosion, though variability exists between individuals (Kimura and Truman, 1990; Lee et al., 2013). It is unknown whether these muscles are intrinsically programed to enter in apoptosis during the early step of adulthood, or conversely whether they receive extrinsic cues. It is known that in *Drosophila* most of the muscles require ecdysone signaling for their degradation (Bate, 1993; Zirin et al., 2013). Exception is made, however, with the male specific muscles of

the A5 segment which depend on neuronal innervation (Bate, 1993). Now that we better understand the wild type pattern of axonal projections of the CCAP motoneurons and their degradation over time, there is no doubt that an attractive experiment to do would be to decrease or to increase *opa* expression in the CCAP neurons and to analyze axonal arborization throughout adult ventral abdomen, with a particular interest on the innervation of the doomed muscles.

Conclusions

1 - In the wing imaginal disc, the Hox gene *Antp* is weakly expressed in the presumptive margin cells and is strongly expressed in the hinge, pleura and notum, especially in the presumptive prescutum.

2 - *Antp* expression in the notum is not related to a homeotic function but is rather involved in thorax closure formation. Removing *Antp* function in the anterior notum leads to a cleft thorax phenotype.

3 - *Antp* regulates thorax closure having an active role in cytoskeleton remodeling and filopodia formation in the leading edge cells. This regulation is probably mediated via JNK signaling, but in a cell death independent manner.

4 - The transcription factor Opa is expressed in the CCAP neurons from embryonic stage to adulthood in the CNS. Its expression starts prior to CCAP hormonal differentiation. Its expression in these cells depends on a DNA fragment of 6 kbp (3R 666795-672762).

5 - Several peripheral CCAP neurons expressing *opa* have been identified as innervating the intestinal tract and the sexual organs of both gender, as well as the aorta and the adult muscles inherited from the larva.

6 - *opa* loss-of-function alters the adult postecdysis behavioral sequence (wing extension, thorax stretching and cuticle tannin) at two levels: it prevents CCAP neurons from cell death and activates *Burs* expression. Opa is therefore the first transcription factor identified as regulator of *Burs* expression.

7 - The gain-of-function of the *opa* homologue gene in vertebrate *Zic2* mimics *opa* gain of function: ectopic expression of *opa* or *Zic2* leads to pupal and adult ecdysis defect.

8 - *opa* gain-of-function is able to activate *Burs* expression in CCAP neurons as well as in no CCAP neurons. *opa* gain-of-function represses *CCAP* expression.

9 - Opa is not involved in CCAP axon guidance during larval stage.

Conclusiones

1 - En el disco imaginal de ala, el gen Hox *Antp* tiene una expresión débil en las células del presuntivo margen y una expresión fuerte en la axila, pleura y notum, especialmente en el futuro prescutum.

2 - La expresión de *Antp* en el notum no está asociada a una función homeótica pero está implicada en el cierre del tórax. La inhibición de la función de *Antp* en el notum anterior da lugar a un tórax adulto hendido.

3 - *Antp* regula el cierre del tórax controlando la remodelación del citoesqueleto y la formación de filopodios en las células del tallo. Este proceso está mediado por la vía de JNK, pero no por la vía de Dpp.

4 - El factor de transcripción Opa se expresa en las neuronas CCAP del sistema nervioso central desde el estadio embrionario hasta el estadio adulto. Su expresión empieza antes de la diferenciación hormonal de las neuronas CCAP. Su expresión en estas células depende de un fragmento de ADN con un tamaño de 6 kpb (3R 666795-672762).

5 - Varias neuronas CCAP del sistema periférico han sido identificadas inervando el sistema circulatorio, el sistema intestinal, los órganos sexuales, y los músculos adultos heredados de la larva. Todas estas neuronas expresan *opa*.

6 - La falta de función de *opa* altera la secuencia de la post-ecdisis adulta (extensión del ala, estiramiento del tórax y el tanino) actuando Opa a dos niveles: impide la muerte de las neuronas CCAP y activa a la expresión de *Burs*. Opa constituye el primer factor de transcripción identificado como regulador de la expresión de *Burs*.

7 - La ganancia de función del homólogo de *opa* en vertebrados, *Zic2,* imita la ganancia de función de *opa*; un incremento de la expresión de *opa* o de *Zic2* altera la ecdisis pupal y adulta.

8 - La ganancia de función de *opa* es capaz activar a *Burs* tanto en las neuronas CCAP como en las neuronas no CCAP. La ganancia de función de *opa* es también capaz de reprimir la expresión de *CCAP*.

9 - Opa no está implicado en la guía axonal de las neuronas CCAP durante el estadio larvario.

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Arthropods cuticle constitutes an external skeleton or exoskeleton. It has a protective function, such as support or defense against predator, but also has a physiologic role by preventing from desiccation or by sensing its environment among others. Arthropods suffer various molting throughout their lifetime. Their cuticle is highly rigid and has to be periodically shed to allow the animal to grow or to change its form. This process highly stereotyped, referred as ecdysis, is followed by a postecdysis period which permits the cuticle to become mature.

Drosophila melanogaster **is a holometabolous insect. Its life cycle starts with the fecundated egg. At the end of the embryogenesis, the embryo hatches to give rise to a small size larva. Two molts are necessary to obtain the grown larva, which then stop moving. The cuticle of the larvae is then shed and used as a cocoon, called pupal case, where a complete metamorphosis (from the Greek *meta* "change" and *morphe* "form") occurs. At the final ecdysis (eclosion), the adult fly emerges. During the post-eclosion behavioral sequence the newly formed and soft cuticle get tanned, that is to say get hardened and darken, and the wings extend.**

Adult thorax derives from a couple of larval structures, which are of ectodermal origin, called wing imaginal discs. During the metamorphosis, they migrate dorsally and fuse at the midline in a two slide fasteners manner similar to a wound healing. During this Thesis, we studied the implication of the Hox gene *Antennapedia* (*Antp*) during thorax closure. We found that *Antp* expression is restricted to the cells that initiate this morphogenetic event and alteration of its expression leads to a split thorax. Our results indicate that this phenotype is associated to a missregulation of JNK signaling which in turn affects actin cytoskeleton organization and filopodia formation needed in the leading edge cells for tissue migration.

We also focus our interest on a later *Drosophila* developmental stage, the post-eclosion. The mechanism underlying the post-eclosion sequence is known to be controlled by the neuropeptide Bursicon, which is synthesized by a subset of neurons called CCAP. We found that the transcription factor Opa is expressed in the CCAP cells and that downregulation of *opa* expression mimics the phenotype of alteration in *Bursicon* expression. Our data point to a requirement of Opa expression in the CCAP cell to prevent them from apoptosis. Besides, we identified Opa as the first uncovered positive regulator of *Bursicon* expression.